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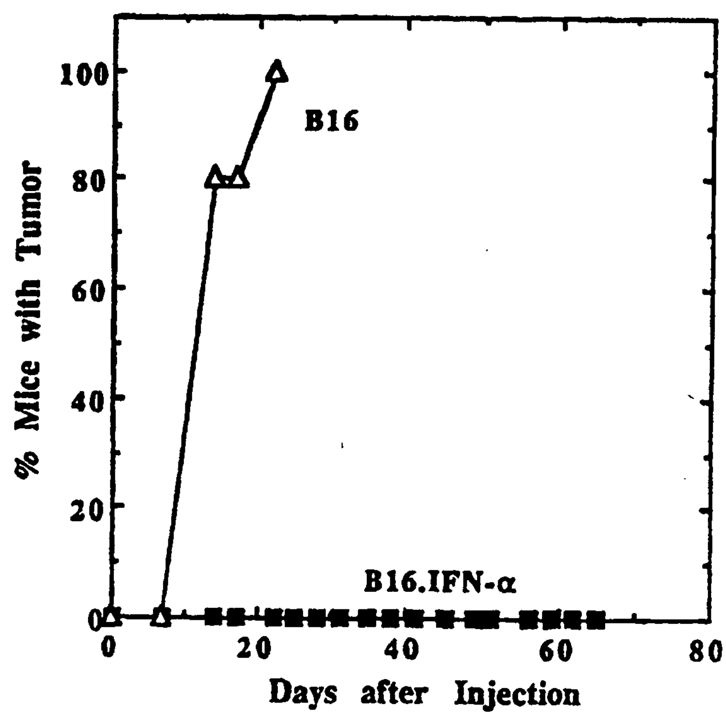
(54) Title: GENE THERAPY OF SOLID TUMORS WITH INTERFERONS ALONE OR WITH OTHER IMMUNO-EFFECTOR PROTEINS

## (57) Abstract

The present invention relates to vectors and compositions for gene therapy strategies against solid tumors, particularly malignant tumors. In one aspect, the invention is directed to a solid tumor vaccine comprising tumor cells transfected to express interferon-$\alpha$ in a pharmaceutically acceptable excipient, and to a method for treating a solid tumor by administering such a vaccine. Preferably, the tumor cells are also transfected to express an immunomodulatory molecule, such as interferon-$\gamma$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, B7-1 T cell costimulatory molecule, B7-2 T cell costimulatory molecule, immune cell adhesion molecule (ICAM)-1 T cell costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof, with the proviso that the immunomodulatory molecule is not interferon-$\alpha$. In yet a further embodiment, a soluble immunomodulatory molecule can be included in a vaccine of the invention. The method comprises introducing into a subject suffering a solid tumor a therapeutically effective number of tumor cells from a solid tumor, which tumor cells are transfected to express interferon-$\alpha$. Preferably, the tumor cells are also transfected to express an immunomodulatory molecule, with the proviso that the immunomodulatory molecule is not interferon-$\alpha$. Alternatively, a therapeutically effective number of additional tumor cells transfected to express an immunomodulatory molecule ca be introduced into the subject.



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# GENE THERAPY OF SOLID TUMORS WITH INTERFERONS ALONE OR WITH OTHER IMMUNO-EFFECTOR PROTEINS

The research leading to this application was supported in part by the United States Public Health Service Grant RO1 CA46465 from the National Cancer Institute. The Government has certain rights in this invention.

## FIELD OF THE INVENTION

The present invention relates to vectors and compositions for gene therapy strategies against solid tumors, particularly malignant tumors.

## BACKGROUND OF THE INVENTION

### IFN$\alpha$ Therapy for Tumors

The discovery of the therapeutic effect of human interferon alpha (IFN-$\alpha$) in hairy cell leukemia, a rare B-cell neoplasm, has led to the emergence of IFN-$\alpha$ as a prototype protein that represses the clinical tumorigenic phenotypes in some malignancies capable of differentiation, (Gutterman JU: 1994, Proc. Natl. Acad. Sci. USA, 91:1198-1205). IFN-$\alpha$ is currently used for the treatment of several cancers including hairy cell leukemia, chronic myelogenous leukemia and Kaposi's sarcoma. However, systemic application of IFN-$\alpha$ is often associated with severe dose-limiting side effects (Quesada JR. et al.: 1986, J. Clin. Oncol. 4:234-244).

Ozzello *et al.* (Ozzello et al.: 1992, Cancer Res. 52:4571-4581) has shown that the intratumoral injection of the combination of IFN-$\alpha$ and IFN-$\gamma$ is more effective in the treatment of cutaneous recurrences in breast carcinoma patients than either cytokine alone. (Balkwill et al., 1986, Cancer Res. 46:3990-3993) showed that systemic application of a combination of IFN-$\alpha$ and TNF-$\alpha$ have antitumor effects on different human tumor xenografts in nude mice.

Many cytokines have been examined for their efficacy in tumor cell-targeted gene therapy in different tumor models (Colombo MP and Forni G, 1994, Immunol.

Today 15(2):48-51). However, apart from one report by (Ferrantini et al., 1993, Cancer Res. 53:1107-1112) using Friends leukemia cells (FLC), derived from committed erythroid stem cells, no studies have been reported regarding the effect of constitutive IFN-$\alpha$ production in solid tumors.

IFN-$\alpha$, produced by mononuclear leukocytes and other cells, is an important regulator of cellular growth and differentiation affecting cellular communication and signal transduction pathways as well as immunological control (Pestka et al. 1987, Ann. Rev. Biochem. 56:727-777); (Lengyl P, 1993, Proc. Natl. Acad. Sci. USA 90:5893-5895). Among the important immunological properties of IFN-$\alpha$ is its ability to enhance major histocompatibility complex (MHC) class I antigen expression and induction of gp96, a heat shock protein associated with antigen presentation (Srivastava PK, 1993, Adv. Cancer Res. 62:153-177).

## IFN-$\gamma$ Therapy for Tumors

In the past few years, a number of laboratories have engineered murine tumor cells to secrete particular cytokines in an attempt to generate potent antitumor responses (reviewed in Colombo and Forni, 1994, Immunol. Today 15:48-51). Upon injection of IFN-$\gamma$-secreting tumor cells into syngeneic mice, it was observed that, depending on the tumor model used and the level of cytokine secreted, the tumorigenic potential of the modified cells was either reduced or completely abrogated as compared to that of the parental cells (Watanabe et al., 1989, Proc Natl. Acad. Sci USA 86:9456-60); (Gansbacher et al., 1990 Cancer Res. 50:7820-7825); (Restifo et al., 1992, J. Exp. Med. 175:1423-1431); (Esumi et al., 1993, Cancer Res. 51:1185-1189); (Lollini et al., 1993, Int. J. Cancer 55:320-329); (Porgador et al., 1993, J. Immunol. 150(4):1458-1470). Although, in general, these studies agreed in that IFN-$\gamma$ gene-transfected tumor cells can be used as a tool to augment immune responses against tumors, some discrepancies were evident regarding the mechanisms involved. Thus, the use of cytokine-gene modified tumor cells as vaccines required further experimentation in order to find ways to improve the observed antitumor responses, to elucidate the immune

mechanisms that are induced in the host, and to facilitate their application to human cancer therapy.

This previous work has failed to ascertain 1) the mechanisms whereby IFN-$\gamma$ induces its antitumor effects *in vivo* and 2) whether co-expression of IFN-$\gamma$ and a costimulatory molecule by solid tumor cells provides the appropriate signals required to activate immune effector cells efficiently. Few reports have been published that describe the effects of cytokine-gene transfer into B16 cells, a spontaneous melanoma tumor line (Fearon et al., 1990, Cell 60:397-403); (Botazzi et al., 1992, J. Immunol. 148(4):1280-1285); (Tepper et al., 1992, Cell 57:503-512); (Sun et al., 1992, Cancer Res. 52:5412-5415); (Dranoff et al., 1993, Proc. Natl. Acad. Sci. USA 90:3539-3543). Moreover, the effects of constitutive secretion of IFN-$\gamma$, or of this cytokine in combination with other molecules such as B7, on the tumorigenicity of a particularly aggressive tumor, such as B16 have not been reported.

B7 T Cell Costimulatory Molecule

The B7 T cell costimulatory molecule is expressed on activated B cells, macrophages and dendritic cells, the so-called professional antigen presenting cells (APC). Upon engagement of the B7 molecules with the CD28 counter-receptors on the lymphocyte cell surface a co-stimulatory signal is transmitted to T helper (Th) cells (Gimmi et al., 1991, Proc. Natl. Acad. Sci. USA 88:6575-6579); (Linsley et al., 1991, J. Exp. Med. 173:721-730); (Nabavi, et al., 1992, Nature 360:266-268). The provision of this secondary signal has been shown to be critical for activation of Th cells along with the specific interaction between the T cell receptor and the peptide/MHC molecule complex (Das Gupta et al., 1987, Proc. Natl. Acad. Sci. USA 84:1094-1098). Moreover, failure to provide both signals results in the development of Th cell anergy. However, nothing is known concerning whether co-expression of IFN-$\gamma$ and B7 by tumor cells will provide the appropriate activation signals required to induce effective antitumor immune mechanisms.

The citation of any reference herein should not be deemed an admission that such reference is available as prior art to the invention.

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## SUMMARY OF THE INVENTION

In one aspect, the invention is directed to a solid tumor vaccine comprising tumor cells transfected to express interferon-$\alpha$ in a pharmaceutically acceptable excipient. Preferably, the tumor cells are also transfected to express an immunomodulatory molecule, with the proviso that the immunomodulatory molecule is not interferon-$\alpha$. Alternatively, additional tumor cells (*i.e.*, cells not transfected with IFN-$\alpha$) are transfected with the immunomodulatory molecule. Examples of such immunomodulatory (or immuno-effector) molecules include, but are not limited to, interferon-$\gamma$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, B7-1 T cell costimulatory molecule, B7-2 T cell costimulatory molecule, immune cell adhesion molecule (ICAM) -1 T cell costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof. Preferably, the tumor cells are from the tumor in the subject. Alternatively, tumor cells from a corresponding or closely related tumor cell line can be used.

In yet a further embodiment, a soluble immunomodulatory molecule can be included in a vaccine of the invention. Examples of such soluble immunomodulatory molecules include, but are not limited to, interferon-$\gamma$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

In addition to the pharmaceutical compositions, the present invention is further directed to a related method for treating a solid tumor. The method comprises introducing into a subject suffering a solid tumor a therapeutically effective number of tumor cells from a solid tumor, which tumor cells are transfected to express interferon-$\alpha$. Preferably, the tumor cells are also transfected to express an immunomodulatory molecule, with the proviso that the immunomodulatory

molecule is not interferon-$\alpha$. Alternatively, a therapeutically effective number of additional tumor cells transfected to express an immunomodulatory molecule can be introduced into the subject.

In a further embodiment, the therapeutic method of the invention comprises introducing an therapeutically effective amount of a soluble immunomodulatory molecule into the subject.

Preferably, the tumor cells are from the tumor in the subject. Alternatively, tumor cells from a corresponding or closely related tumor cell line can be used. More preferably, the tumor cells are introduced in proximity of the tumor in the subject.

In a further related aspect, the invention is directed to a method for treating a solid tumor comprising introducing into a subject suffering from a solid tumor an expression vector directed to cells of the solid tumor, which expression vector codes on expression for interferon-$\alpha$. Preferably, the method further comprises introducing into the subject a second expression vector directed to cells of the solid tumor, which second expression vector codes on expression for an immunomodulatory molecule. In an alternative preferred embodiment, the expression vector directed to cells of the solid tumor additionally codes on expression for an immunomodulatory molecule.

In another aspect, the present invention is directed to a solid tumor vaccine comprising tumor cells transfected to express interferon-$\gamma$ and an immunomodulatory molecule, in a pharmaceutically acceptable excipient. Alternatively, additional tumor cells transfected to express an immunomodulatory molecule, with the proviso that the immunomodulatory molecule is not interferon-$\gamma$. Examples of immunomodulatory molecules include but are not limited to interferon-$\alpha$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15,

B7-1 T cell costimulatory molecule, B7-2 T cell costimulatory molecule, immune cell adhesion molecule (ICAM) -1 T cell costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof. As can be appreciated, the immunomodulatory molecule can be expressed by the tumor cells transfected with IFN-$\gamma$, by additional tumor cells not transfected with IFN-$\gamma$, but only transfected with the immunomodulatory molecule, or by addition of a soluble immunomodulatory molecule.

Thus, in another embodiment, the solid tumor vaccine of this aspect of the invention further comprises a soluble immunomodulatory molecule, *e.g.*, interferon-$\alpha$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

Similarly, the invention provides a method for treating a solid tumor comprising introducing into a subject suffering the solid tumor a therapeutically effective number of tumor cells from a solid tumor, which tumor cells are transfected to express interferon-$\gamma$ and an immunomodulatory molecule, with the proviso that the immunomodulatory molecule is not interferon-$\gamma$. Alternatively, the method of the invention comprises introducing a therapeutically effective number of additional tumor cells transfected to express an immunomodulatory molecule into the subject. As noted above, the tumor cells can be transfected both with IFN-$\gamma$ and an immunomodulatory molecule, or separately with IFN-$\gamma$ and the immunomodulatory molecule. In addition, the method of the invention may involve introducing the therapeutically effective amount of a soluble immunomodulatory molecule into the subject.

Preferably, the tumor cells are from the tumor in the subject. Alternatively, a related or similar tumor cell line can be used. In another preferred embodiment, the tumor cells are introduced in proximity of the tumor in the subject.

In a related aspect, the invention provides a method for treating a solid tumor comprising introducing into a subject suffering from a solid tumor an expression vector directed to cells of the solid tumor, which expression vector codes on expression for interferon-$\gamma$, and introducing into the subject a second expression vector directed to cells of the solid tumor, which second expression vector codes on expression for an immunomodulatory molecule, with the proviso that the immunomodulatory molecule is not interferon-$\gamma$. In another embodiment, the method for treating a solid tumor comprises introducing into a subject suffering from a solid tumor an expression vector directed to cells of the solid tumor, which expression vector codes on expression for interferon-$\gamma$ and an immunomodulatory molecule, with the proviso that the immunomodulatory molecule is not interferon-$\gamma$.

Thus, the present invention is directed toward treatment and therapy of solid tumors in a subject. Preferably, the subject is a mammal, and more preferably, a human. In specific embodiments, *infra*, the subject is a mouse suffering from melanoma.

## BRIEF DESCRIPTION OF THE DRAWINGS

**FIGURE 1. Cytofluorographic analysis of MHC class I and class II expression on B16 parental and IFN-$\gamma$ cDNA-transfected cells.** Expression of MHC class I and class II molecules was determined as described in "Materials and Methods", in Example 1. Briefly, 1 x $10^6$ B16 parental (panel A) or B16.IFN-$\gamma$ cells (panel B) were incubated with 50 $\mu$l of culture supernatant containing MAb 28-8-6S (anti H-$2K^b$) or MAb AF6-120.1.2 (anti-I-$A^b$) at 4°C for 30 min. After washing with medium containing 10% FBS, cell pellets were stained by incubation with goat anti-mouse FITC-conjugated secondary antibody (80 $\mu$g/ml) at 4°C for 30 min. Cell-surface staining was analyzed on a Coulter Epics-Profile II cytofluorograph. The fluorescence values shown are not linear but represent the fluorescence

detection channels of the cytofluorograph. The real X-axis values span approximately three decades of a logarithmic scale.

**FIGURE 2. Cytofluorograph in analysis of B7 expression on B7 cDNA-transfected cells.** Expression of B7 molecules was determined as described in "Materials and Methods" (Example 1, *infra*). Briefly, 1 x $10^6$ B16 parental (A), B16.B7.7 (B), or B16.IFN-$\gamma$/B7.11 (C) cells were incubated with 50 $\mu$l of culture supernatant containing MAb 1G10-G9 (anti-mouse B7) at 4°C for 30 min. After washing with medium containing 10% FBS, cell pellets were stained by incubation with goat anti-rat FITC-conjugated secondary antibody (80 $\mu$g/ml) at 4°C for 30 min. Cell-surface staining was analyzed on a Coulter Epics-Profile II cytofluorograph after washing and fixing the cells with 1% paraformaldehyde in PBS. Dotted line represents staining with FITC-conjugated antibody only (background), solid line represents B7 expression. The fluorescence values shown are not linear but represent the fluorescence detection channels of the cytofluorograph. The real X-axis values span approximately three decades of a logarithmic scale.

**FIGURE 3. Tumorigenicity of B16 parental and B16.IFN-$\gamma$ cells in syngeneic mice.** B16 parental or B16.IFN-$\gamma$ cells were injected s.c. into 6-8 weeks old male syngeneic C57BL/6J mice in 0.2 ml HBSS. As a control, a group of mice was injected with B16 cells transfected with vector only (B16.pD5neo). Animals were monitored twice a week by palpation of the injection site over a period of 100 days. Panel A: Percentage of mice with a palpable tumor at various days after injection of 1 x $10^5$ B16 parental or B16.IFN-$\gamma$ cells. Panel B: Percentage of mice with a palpable tumor at various days after injection of 1 x $10^6$ B16 parental, B16.IFN-$\gamma$, or B16.pD5neo cells.

**FIGURE 4. Tumorigenicity of B16.B7, B16.IFN-$\gamma$/B7 and a mixture of B16.B7 and B16.IFN-$\gamma$ cells.** B16.B7, B16.IFN-$\gamma$/B7, and a combination of B16.B7 and B16.IFN-$\gamma$ cells were injected s.c. into syngeneic C57BL/6J mice at 1

x $10^6$ cells/mouse. Parental B16 and B16.IFN-$\gamma$ cells were also injected as controls. Mice were monitored twice a week by palpation of the injection sites. Animals with massive tumor burden were sacrificed for humane reasons. Percentage of mice with a palpable tumor at various days after injection of 1 x $10^6$ B16 parental, B16.IFN-$\gamma$, B16.B7, B16.IFN-$\gamma$/B7, or after injection of a combination of B16.IFN-$\gamma$ and B16.B7.7 cells (1 x $10^6$ cells each) are shown.

**FIGURE 5. Tumorigenicity of B16 parental and cytokine-secreting cells in syngeneic C57/BL6 mice.** Mice were injected with either 1 x $10^6$ B16 parental (5 mice) or B16.IFN-$\alpha$ (10 mice) cells subcutaneously on the flank. Tumor development was monitored twice a week by palpation of the injection site.

**FIGURE 6. Tumorigenicity of a combination of B16.IFN-$\alpha$ and B16.IFN-$\gamma$ cells.** C57BL/6 mice were injected with 1 x $10^6$ B16 (5 mice), B16.IFN-$\gamma$ (5 mice), B16.IFN-$\alpha$ (14 mice) and a combination of B16.IFN-$\alpha$ and B16.IFN-$\gamma$ (20 mice) injected s.c. in the flank. Tumor development was monitored as described under "Materials and Methods" in Example 2.

**FIGURE 7. Tumorigenicity of a combination of B16.IFN-$\alpha$ and B16.TNF-$\alpha$ cells.** C57BL/6 mice were injected with 1 x $10^6$ B16.TNF-$\alpha$ cells (5 mice), combination of B16.IFN-$\alpha$ and B16.TNF-$\alpha$ cells (1 x $10^6$ each; 10 mice) and B16.IFN-$\alpha$ cells on the right flank and B16.TNF-$\alpha$ cells on the left flank (1 x $10^6$ cells; 10 mice).

**FIGURE 8. Tumorigenicity of a combination of B16 and B16.IFN-$\alpha$ cells in a mixed tumor transplantation assay.** C57BL/6 mice were injected with 1 x $10^6$ B16 parental cells (5 mice), B16.IFN-$\alpha$ (14 mice) or a combination of B16 parental and B16.IFN-$\alpha$ cells (1 x $10^6$ each; 10 mice). Tumor development was monitored as described under "Materials and Methods" in Example 2.

**FIGURE 9. Local curative p tential of irradiated B16.IFN-$\alpha$ cells.** Mice were injected with 1 x $10^5$ B16 parental cells. Beginning three days after the injection of the tumor inoculum, mice were injected 3 times at weekly interval with 5 x $10^6$ irradiated B16.IFN-$\alpha$ cells at the tumor site. Tumor growth was compared with the control group which received only the parental cells but no irradiated B16.IFN-$\alpha$ cells. Ten mice were injected with 1 x $10^5$ B16 cells. Three days later, 5 mice were treated with irradiated B16.IFN-$\alpha$ cells as described above. There was no difference in IFN-$\alpha$ secretion between irradiated and non-irradiated cells.

**FIGURE 10. Local curative potential of irradiated B16.IFN-$\alpha$ cells.** The experiment was performed as described in the legend to FIGURE 9 except that the tumors were permitted to establish for 7 days prior to initiating the injections of the B16.IFN-$\alpha$ cells. Thirteen mice were injected with 1 x $10^5$ B16 cells. Seven of them were treated with irradiated B16.IFN-$\alpha$ cells as described in the legend to FIGURE 9.

**FIGURE 11. Plasmid pD5 used for construction of the mouse IFN-$\gamma$ expression vector.**

**FIGURE 12. Expression vector for cotransfection.** This vector contains the neomyocis phosphotransferase gene (*neo*) under control of the phosphoglycerol lunase promoter (pPpk). It was used in contranfection experiments where the expression plasmid for IFN-$\gamma$ or other factors did not contain the *neo* gene.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides effective vaccines for solid tumor cancers. In particular, the invention represents the first time interferon-$\alpha$ (IFN-$\alpha$) has shown activity in abrogating tumorigenicity. The present inventors have also provided a much more effective use of interferon-$\gamma$, and were able to completely abrogate

tumorigenicity (as opposed to merely delaying tumor growth) by transfecting tumor cells with IFN-$\gamma$ and another immunomodulatory molecule, specifically, B7 T cell costimulatory molecule.

As used herein, the term "solid tumor" and "tumor" refer to a neoplasm, *i.e.*, tissue that grows by cellular proliferation more rapidly than normal, *e.g.*, more rapidly than adjoining cells, or other cells in the tissue. Neoplastic cells continue to grow after growth stimuli cease. Generally, tumors represent or form a distinct mass of tissue. A tumor may be benign or malignant (cancer). The present invention relates to both types of tumors, but is particularly valuable in the treatment of cancers. Various type of solid tumors are described in detail, *infra.*

Tumor cells are the neoplastic cells from the tumor. For purposes of the present invention, such cells are part of the living cells in the tumor, so that they can be transfected to express either IFN-$\alpha$, or IFN-$\alpha$ with another immunostimulatory molecule; or IFN-$\gamma$ with another immunostimulatory molecules. In a preferred aspect of the invention, tumor cells from a subject suffering from a solid tumor are obtained, *e.g.*, by biopsy, and these cells are transfected. However, the invention also contemplates use of tumor cell lines that correspond to or sufficiently emulate an *in vivo* tumor transfected with an $\alpha$ or $\gamma$ IFN, and possibly with another immunomodulatory molecule.

The term "immunomodulatory molecules" as used herein refers to lymphokines, cytokines, cellular adhesion molecules associated with immune responses, *i.e.*, any molecule that mediates immunostimulation, immunosuppression, specific immune recognition, and the like. According to the invention, the immunomodulatory molecules are in addition to either IFN-$\gamma$ or IFN-$\alpha$, and include the other IFN ($\gamma$ or $\alpha$, respectively), and IFN-$\beta$; cytokines such as but not limited to interleukin (IL) 1, IL-2, IL-4, IL-6, IL-7, IL-12, tumor necrosis factor (TNF) $\alpha$, TNF-$\beta$, granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage CSF (GM-CSF) (*see, e.g.*, Howar et al., In Fundamental Immunology, 3rd Edition, W.

Paul (editor), pp.763-800; Durum and Oppenheim, ibid., pp. 801-835); accessory molecules, including members of the integrin superfamily and members of the Ig superfamily such as, but not limited to, LFA-1, LFA-3, CD22, and B7-1, B7-2, and ICAM-1 T cell costimulatory molecules (*see* Shevach, *ibid.*, pp. 531-575). In the Examples, *infra*, IFN-$\gamma$ and TNF-$\alpha$ are used as immunomodulatory molecules in addition to IFN-$\alpha$. In another Example, B7 is used in addition to IFN-$\gamma$. The immunomodulatory molecules of the invention are well characterized, and generally human and murine homologs are available. Thus, the present invention contemplates use of clones of such immunomodulatory molecules, or independently generating such clones by well known techniques of molecular biology, using the DNA sequence information available in the art for these molecules.

The present invention provides for *ex vivo* gene transfer of IFN-$\alpha$ or IFN-$\gamma$, possibly with another immunomodulatory molecule, into tumor cells, followed by introduction of those tumor cells into a subject. Alternatively, *in vivo* gene transfer, with gene transfer targeted to tumor cells, can be employed to transpect the tumor cells with a vector or vectors capable of expressing IFN$\gamma$ and another immunomodulatory molecule.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame

oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15 percent, preferably by at least 50 percent, more preferably by at least 90 percent, and most preferably prevent, solid tumor growth. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host, *e.g.*, a reduction in size of a solid tumor.

According to the invention, the component or components of a therapeutic composition of the invention may be introduced parenterally, *e.g.*, via intravenous injection, and also including, but is not limited to, intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial administration. More preferably, a vaccine of the invention, or an expression vector, may be introduced by injection into the tumor or into tissues surrounding the tumor.

A subject in whom gene therapy treatment of a solid tumor is appropriate is preferably a human, but can be any animal. Thus, as can be readily appreciated by one of ordinary skill in the art, the compositions and methods of the present invention are particularly suited to treatment of any animal, particularly a mammal, and including, but by no means limited to, domestic animals, such as feline or canine subjects, farm animals, such as but not limited to bovine, equine, caprine, ovine, and porcine subjects, wild animals (whether in the wild or in a zoological garden), research animals, such as mice, rats, rabbits, goats, sheep, pigs, dogs, cats, etc., *i.e.*, for veterinary medical use.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual,* Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach,* Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "cassette" refers to a segment of DNA that can be inserted into a vector at specific restriction sites. The segment of DNA encodes a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation.

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The term transfection includes the revers transcription of viral RNA into DNA. A cell has been "transformed" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change. Preferably, the transforming DNA should be integrated (covalently linked) into chromosomal DNA making up the genome of the cell.

"Heterologous" DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell.

A "clone" is a population of cells derived from a single cell or common ancestor by mitosis.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules") in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5′ to 3′ direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

"Homologous recombination" refers to the insertion of a foreign DNA sequence of a vector in a chromosome. Preferably, the vector targets a specific chromosomal site for homologous recombination. For specific homologous recombination, the vector will contain sufficiently long regions of homology to sequences of the chromosome to allow complementary binding and incorporation of the vector into the chromosome. Longer regions of homology, and greater degrees of sequence similarity, may increase the efficiency of homologous recombination.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3′ to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3′ terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced and translated into the protein encoded by the coding sequence.

A "signal sequence" is included at the beginning of the coding sequence of a protein to be expressed on the surface of a cell. This sequence encodes a signal peptide, N-terminal to the mature polypeptide, that directs the host cell to translocate the polypeptide. The term "translocation signal sequence" is used herein to refer to this sort of signal sequence. Translocation signal sequences can be found associated with a variety of proteins native to eukaryotes and prokaryotes, and are often functional in both types of organisms.

The present invention is based in part on experiments using gene therapy techniques for the treatment of a model melanoma in mice. In the first experiment, B16 melanoma cells were transfected with IFN-$\gamma$, or IFN-$\gamma$ and B7. B16 melanoma was chosen as the tumor model because it is a poorly immunogenic tumor that arose spontaneously in C57BL/6 mice (Fidler, 1975, Cancer Res., 35:218-224) and thus it is more akin to human cancer than most of the mouse tumor models that have been studied. It was found that while transfection of IFN-$\gamma$ alone delayed tumorigenicity, it failed to completely abrogate tumorigenicity. However, administration of tumor cells transfected with both IFN-$\gamma$ and B7 dramatically suppressed tumorigenicity. These results demonstrated that vaccines prepared from tumor cells that express IFN-$\gamma$ and another immunomodulatory molecule, particularly B7, may be effective as a protective vaccine against an existing tumor, or to prevent establishment of a tumor.

The invention is further based on the unexpected discovery that IFN-$\alpha$ can be effective in abrogating solid tumorigenicity. In particular, experiments were performed to explore the antitumor potential of IFN-$\alpha$ when autologous tumor cells transfected with cDNA encoding IFN-$\alpha$ are used as a tumor vaccine to induce a potent immune response against unmodified tumor cells. In this experiment, the B16 melanoma cells were employed. The experiment was further designed to investigate whether a combination of IFN-$\alpha$ and other cytokines such as IFN-$\gamma$ or TNF-$\alpha$ can enhance the antitumor response since the effects of IFN-$\alpha$ are known to be synergized by IFN-$\gamma$ or TNF-$\alpha$ (Fleischmann WR and Schwartz

LA, 1986, Methods Enzymol. 79:432-440; Fiers W, 1991, FEBS Letters 285(2):199-212). The results of this experiment represent the first time IFN-$\alpha$ has been found to be effective against solid tumors.

## Expression Vectors for Immunomodulatory Molecules

The nucleotide sequence coding for an immunomodulatory molecule, or pharmaceutically active fragment or analog thereof, can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Such elements are termed herein a "promoter." Thus, the nucleic acid encoding an immunomodulatory molecule of the invention is operationally associated with a promoter in an expression vector of the invention. Both cDNA and genomic sequences can be cloned and expressed under control of such regulatory sequences. The necessary transcriptional and translational signals can be provided on a recombinant expression vector, or they may be supplied by the native gene encoding immunomodulatory molecule and/or its flanking regions.

For effective expression and therapeutic effect, the recombinant immunomodulatory molecule of the invention is expressed chromosomally, after integration of the coding sequence by recombination. In this regard, any of a number of amplification systems may be used to achieve high levels of stable gene expression (*See* Sambrook et al., 1989, *supra*).

Expression of immunomodulatory molecules may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host tumor cells in which expression is desired. Promoters which may be used to control immunomodulatory molecule gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3′ long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797),

human cytomegalovirus (CMV) promoter, the adenovirus major late promoter, the the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); and animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378). In specific examples, *infra*, the human CMV and adenovirus major late promoters are employed for expression of IFN-$\alpha$ and B7 and IFN-$\gamma$, respectively.

Once a particular recombinant DNA expression vector for use in the invention is prepared, several methods known in the art may be used to propagate it. Once a

suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus, and plasmid and cosmid DNA vectors, to name but a few.

Vectors are introduced into the desired host cells by methods known in the art, *e.g.*, *ex vivo* viral vectors, particularly retroviral vectors, *in vivo* viral vectors, particularly defective viral vectors or adeno-associated virus vectors, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (see, *e.g.*, Wu et al., 1992, J. Biol. Chem. 267:963-967; Wu and Wu, 1988, J. Biol. Chem. 263:14621-14624; Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990).

<u>Gene Therapy Techniques</u>

A nucleic acid coding sequence encoding an immunomodulatory molecule may be introduced *in vitro* or, more preferably, *in vivo* in a viral vector. Such vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. Defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, a solid tumor can be specifically targeted. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector (Kaplitt et al., 1991, Molec. Cell. Neurosci. 2:320-330), an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al. (1992, J. Clin. Invest. 90:626-630), and a defective adeno-associated virus vector (Samulski et al., 1987, J. Virol. 61:3096-3101; Samulski et al., 1989, J. Virol. 63:3822-3828).

In another embodiment the gene can be introduced in a retroviral vector, *e.g.*, as described in Anderson et al., U.S. Patent No. 5,399,346; Mann et al., 1983, Cell 33:153; Temin et al., U.S. Patent No. 4,650,764; Temin et al., U.S. Patent No. 4,980,289; Markowitz et al., 1988, J. Virol. 62:1120; Temin et al., U.S. Patent No. 5,124,263; International Patent Publication No. WO 95/07358, published March 16, 1995, by Dougherty et al.; and Kuo et al., 1993, Blood 82:845. Retroviral vectors are especially attractive for transfecting solid tumors, since the cells of the tumor are replicating.

Alternatively, the vector can be introduced *in vitro* or *in vivo* by lipofection. For the past decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids *in vitro*. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner, et. al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:7413-7417; *see* Mackey, et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:8027-8031)). The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes (Felgner and Ringold, 1989, Science 337:387-388). The use of lipofection to introduce exogenous genes into the specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells, in this instance tumor cells, *e.g.*, via tumor-specific cell surface receptors, represents one area of benefit. Lipids may be chemically coupled to other molecules for the purpose of targeting (*see* Mackey, et. al., 1988, *supra*). Targeted peptides, *e.g.*, hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

It is also possible to introduce the vector *ex vivo* or *in vivo* as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate

precipitation, use of a gene gun, or use of a DNA vector transporter (see, *e.g.*, Wu et al., 1992, J. Biol. Chem. 267:963-967; Wu and Wu, 1988, J. Biol. Chem. 263:14621-14624; Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990).

## Solid Tumors

As noted above, the present invention is directed to generating endogenous immune responses against solid tumors. To date, there has been no effective IFN-$\alpha$-based treatment of solid tumors, and IFN-$\gamma$-based treatments of solid tumors delay, but do not appear to prevent, tumor growth. The vaccines and methods of the present invention overcome these deficiencies, and are applicable to treatment of a wide range of solid tumors.

Examples of solid tumors that can be treated according to the invention include sarcomas and carcinomas such as, but not limited to: fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma.

In another embodiment, dysproliferative changes (such as metaplasias and dysplasias) are treated or prevented in epithelial tissues such as those in the cervix, esophagus, and lung. Thus, the present invention provides for treatment of conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred (for review of such abnormal growth conditions, see Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-79). Hyperplasia is a form of controlled cell proliferation involving an increase in cell number in a tissue or organ, without significant alteration in structure or function. As but one example, endometrial hyperplasia often precedes endometrial cancer. Metaplasia is a form of controlled cell growth in which one type of adult or fully differentiated cell substitutes for another type of adult cell. Metaplasia can occur in epithelial or connective tissue cells. Atypical metaplasia involves a somewhat disorderly metaplastic epithelium. Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia; it is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply stained nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs where there exists chronic irritation or inflammation, and is often found in the cervix, respiratory passages, oral cavity, and gall bladder. For a review of such disorders, see Fishman et al., 1985, *Medicine*, 2d Ed., J. B. Lippincott Co., Philadelphia. The present invention is advantageously suited to intervention at the stage of dysproliferative changes, before a condition proceeds to status as a full blown tumor.

In specific embodiments, *infra*, the present invention is employed in the treatment of a melanoma tumor.

Treatment of other hyperproliferative disorders is also contemplated.

The present invention may be better understood by reference to the following examples, which are provided by way of exemplification and are not intended to limit the scope of the invention.

EXAMPLE 1: IFN-$\gamma$ and B7 as Costimulators of Antitumor Immune Responses

Mouse IFN-$\gamma$ and/or the mouse B7 (T cell costimulatory molecule) cDNAs were transfected into B16 melanoma cells to study the effects of local constitutive expression of these molecules on the tumorigenicity and immunogenicity of this aggressive tumor. Cells expressing IFN-$\gamma$ (B16.IFN-$\gamma$), B7 (B16.B7), B7 and IFN-$\gamma$ (B16.IFN-$\gamma$/B7), and parental cells were injected subcutaneously (s.c.) into syngeneic C57BL/6 mice to compare their *in vivo* growth. IFN-$\gamma$ secretion significantly reduced the tumorigenicity of B16 cells. These effects were related to the direct action of secreted IFN-$\gamma$ since i) *in vivo* injection of antiserum to IFN-$\gamma$ accelerated tumor growth, ii) development of tumor correlated with loss of IFN-$\gamma$ production, and iii) B16.IFN-$\gamma$ cells were tumorigenic in IFN-$\gamma$ receptor (IFN-$\gamma$R) knockout mice, but not in parental mice. These results indicate that immune mechanisms are being activated by IFN-$\gamma$ since i) immune effector cells were recruited to the injection site, ii) expression of MHC class I and class II antigens was increased on cells secreting IFN-$\gamma$ and, iii) B16.IFN-$\gamma$ tumors appeared earlier in athymic mice than in immunocompetent mice. Since the *in vivo* growth of B16.IFN-$\gamma$ cells was not completely abolished, the effect of co-expression of IFN-$\gamma$ and the T cell costimulatory molecule B7 on the tumorigenicity of B16 cells was studied. B16.IFN-$\gamma$/B7 cells, which also express increased levels of MHC class I and class II molecules as compared to parental cells, had a dramatically suppressed tumorigenicity, while B16 cells expressing the B7 molecule only (B16.B7) were as tumorigenic as the parental cells. B16.IFN-$\gamma$/B7 cells induced specific immune responses since all of the protected mice were able to reject challenges with parental cells. These results indicate that co-expression of two molecules involved in the activation of immune responses and in antigen presentation can influence the ability of the immune system to recognize

and eliminate both transfected as well as parental tumor cell inocula, and suggest that vaccines consisting of such cells may be used for the immunotherapy of cancer.

## Materials and Methods

**Mice.** Male C57BL/6J and C57BL/6J-athymic mice, 6-8 weeks old, were obtained from The Jackson Laboratory (Bar Harbor, ME). The parental 129/SvEv wild type and IFN-γR knockout mice were described (Huang et al., 1993, Science 259:1742-1745).

**Cells.** B16 melanoma cells, are melanocytic tumor of C57BL/6 origin (Fidler, 1975, Cancer Res. 35:218-224). B16 cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 500 μg/ml gentamicin sulfate in a humidified incubator supplemented with 5% $CO_2$ at 37°C. Prior to use, cell monolayers were washed with phosphate-buffered saline (PBS) and detached by incubating at 37°C with 2 mM EDTA in PBS.

**Expression Vectors for Tranfection.** A 1.5 kb *Sau*3A fragment containing the complete coding sequence of the murine IFN-γ cDNA from the plasmid pMifko-1 (Gen BANK Accession No. K00083 for murine immune IFN (IFN-γ) DNA; Nabori et al., 1992, Nature 360:266-268) was subcloned into the *Bam*HI site of the eukaryotic expression vector pD5 (Figure 11) downstream from the adenovirus major late promoter. The pD5-IFN-γ construct, as well as the expression vector alone, were used for transfection into B16 cells along with plasmid pPGKneobpA (Figure 12), which contains the bacterial neomycin resistance gene under control of the phosphoglycerol kinase promoter. The eukaryotic expression vector pRc/neoCMV-MB7 contains the complete coding sequence of murine B7 subcloned downstream from the CMV promoter and the bacterial neomycin resistance gene under the control of the SV40 early promoter. The coding region

for the mouse B7 protein (Baskar et al., 1993, inserted into the *Ava* I/*Not* I sites of the RC/CMV expression vector (Invitrogen).

**DNA transfection.** Twenty $\mu$g of plasmids pD5-IFN-$\gamma$ or pD5 and 2 $\mu$g of plasmid pPGKneobpA were co-transfected into 5 x $10^5$ B16 cells by the calcium-phosphate precipitation method (Chen and Okayama, 1988, Mol. Cell. Biol. 7(8):2745-2752) followed by selection and cloning in DMEM containing the neomycin analogue G418 (Geneticin; Gibco) at a concentration of 1 mg/ml. Clones were maintained in medium containing antibiotic G418 at 500 $\mu$g/ml. The pRc/neoCMV-mB7 construct (20 $\mu$g) was transfected into B16 melanoma cells by the calcium-phosphate precipitation method. Selection of antibiotic G418 resistant clones was carried out as described. Twenty $\mu$g of plasmid pRc/neoCMV-mB7 were also co-transfected into B16.IFN-$\gamma$ cells along with 1 $\mu$g of plasmid pHM24 an *E. coli* plasmid containing the hygromycin gene driven by a eukaryotic promoter (Koo et al., 1994, Virology 205:345-351; Mikawa et al., 1991, Exp. Cell Res. 195:516-523; Brown and Scott, 1987, in *DNA Cloning-A Practical Approach, Volume 3*, DM Glove (editor) IRL Press; Oxford, Washington, D.C., p. 189), which contains a gene conferring resistance to the antibiotic hygromycin B. Transfection was performed by the polybrene-DMSO method as described in Sambrook *et al.* (1982). After twenty-four hours at 37°C, selection medium containing 1 mg of antibiotic G418/ml and 200 $\mu$g of hygromycin B/ml (Calbiochem) was added. Antibiotic G418- and hygromycin B-resistant clones were assayed for expression of both IFN-$\gamma$ and B7 by antiviral assay and flow cytometry, respectively. Positive clones expressing the B7 molecule and IFN-$\gamma$, and containing both neomycin and hygromycin markers, were maintained in DMEM containing 500 $\mu$g antibiotic G418/ml and 100 $\mu$g hygromycin B/ml.

**Interferon assay.** Antibiotic G418-resistant clones were assayed for the production of IFN-$\gamma$ by the cytopathic effect inhibition assay with encephalomyocarditis virus (EMCV) on mouse L929 cells (Familletti et al., 1981, Methods Enzymol. Pestka S (ed.) New York, Academic Press, 78:430-435).

Antibiotic G418-resistant clones were seeded in 6-well tissue culture plates at 1 x $10^6$ cells per well in 1 ml DMEM containing 10% FBS without antibiotic G418. After 48 hours, culture supernatants were removed, then serially diluted 2-fold in 96-well microtiter plates followed by addition of 4 x $10^4$ L929 cells per well. After overnight incubation at 37°C, EMCV was added and the incubation continued for another 24 hours after which plates were stained with crystal violet to observe viable cells. IFN activity was expressed in units/ml calibrated against the NIH reference standard Gg 02-901-533 for mouse IFN-$\gamma$.

**Hybridomas and antibodies.** Hybridoma 28-8-6S secreting a mouse anti-H-$2K^b$ (class I) monoclonal antibody has been described (Ozato et al., 1985, Cancer Res. 52:4571-4581). Hybridoma AF120.1.2 producing a mouse anti-I-$A^b$ (class II) monoclonal antibody was obtained from the American Type Culture Collection (Loken and Stall, 1982, J. Immunol. Meth. 50:R85). Hybridoma 1G10-G9 secreting a rat anti-mouse B7 monoclonal antibody has been described (Leo et al., 1987, Proc. Natl. Acad. Sci. USA 84:1374-1380). Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse or anti-rat immunoglobulin (Cappel) were used as secondary antibodies in flow cytometry analysis of cell surface expression. Hybridomas were seeded at 1 x $10^5$ cells/ml in DMEM supplemented with 10% FBS, 10 mM HEPES, 1 x L-glutamine, 1 x non-essential amino acids, 10% NCTC-135 medium (Gibco) and 1 x of Solution I [1.32 g oxaloacetic acid (100 mM), 80 mg bovine insulin (20 units/ml, 25 units/mg), 550 mg sodium pyruvate (50 mM) in a volume of 100 ml]. Incubations were carried out at 37°C in an atmosphere of 5% $CO_2$ until the cultures reached confluence. Culture supernatants were centrifuged at 4000 rpm on a table-top centrifuge for 20 min to pellet the cells, then filtered to eliminate remaining cells and maintain sterility. Clarified supernatants were maintained at -20°C until used.

**Flow cytometry.** Expression of cell surface molecules was assayed by incubation of cells with specific monoclonal antibodies (MAb) followed by incubation with fluorescein isothiocyanate labeled (FITC)-goat anti-mouse or goat anti-rat

immunoglobulin at a concentration of 80 $\mu$g/ml. Parental B16 cells or B16 cells expressing mouse IFN-$\gamma$ (1 x $10^6$) were resuspended in 50 $\mu$l of culture supernatant containing MAb 28-8-6S (anti-H-$2K^b$) or MAb AF6-120.1.2 (anti I-$A^b$), then incubated at 4°C for 30 min. After washing the cells thoroughly with complete medium, cells were stained by incubation with goat anti-mouse FITC-labeled secondary antibody for another 30 min at 4°C. Expression of the transfected B7 molecule was analyzed by incubation with MAb 1G10-G9 (anti-mouse B7) followed by incubation with goat anti-rat FITC-labeled secondary antibody as described (Leo *et al.*, 1987). After washing twice with complete medium and once with PBS, cells were fixed with 1% paraformaldehyde in PBS. Cell-surface staining of MHC class I and class II molecules as well as that of the B7 molecule was analyzed on a Coulter Epics-Profile II cytofluorograph as previously described (Jung et al., 1988, Som. Cell and Molec.Gen. 14:583-592); (Cook et al., 1992, J. Biol. Chem., 269:7013-7018. ).

***In vitro* cell growth.** To assess whether IFN-$\gamma$ production affects the growth of B16.IFN-$\gamma$ cells we performed *in vitro* growth assays. B16 and B16.IFN-$\gamma$ cells were seeded at 1 x $10^5$ cells per well in 6-well tissue culture plates and incubated at 37°C. On days 1, 2 and 3 cells were counted with a hemocytometer after staining with trypan blue to exclude dead cells.

***In vivo* tumor growth.** C57BL/6J male mice were injected subcutaneously (s.c.) on the flank with various numbers of B16, B16.IFN-$\gamma$, B16.B7, or B16.IFN-$\gamma$/B7 cells in 0.2 ml of Hank's balanced saline solution (HBSS). C57BL/6J-athymic mice, 129/SvEv wild type and 129/SvEv mice lacking the IFN-$\gamma$ receptor gene (knockout mice) were injected with parental or B16.IFN-$\gamma$ cells as described above. Tumor growth was followed by palpation and recorded as the average of two-dimensional caliper measurements in cm. Mice that rejected the primary injection of tumor cells were challenged s.c. on the opposite flank with parental B16 cells. Animals with massive tumor burden were sacrificed to avoid undue discomfort.

**Effect of anti-IFN-γ polyclonal serum.** C57BL/6J mice were injected s.c. with 1 x $10^6$ B16.IFN-γ cells and intraperitoneally (i.p.) with a rabbit polyclonal antiserum raised against murine IFN-γ (5 x $10^4$ neutralization units). As controls, one group of mice was injected with non-specific rabbit serum and another group was injected only with cells. Tumor growth was followed as described above.

**Histological analysis of tumor tissue.** Tissue at the site of injection of B16 parental and B16.IFN-γ cells was obtained at various times and fixed in 10% phosphate-buffered formalin (22 mM $NaH_2PO_4$, 46 mM $Na_2HPO_4$, 10% formalin). Paraffin-embedded sections were analyzed by conventional histological staining for evaluation of the tumors and of the inflammatory cell infiltrate as described (Ozzello et al., 1992, Cancer Res. 52:4571-4581).

***In vitro* culture of tumor cells.** Tissue from B16.IFN-γ tumors was minced to obtain single cell suspensions which were incubated in DMEM containing 10% FBS at 37°C. After several passages, culture supernatants were assayed for the presence of IFN-γ by the interferon assay described above.

**Southern and Northern analysis.** DNA and RNA were extracted from cultured B16 cells, B16.IFN-γ cells as well as from B16.IFN-γ tumors (Sambrook et al., 1989, supra; Kumar et al., 1989, J. Biol. Chem. 264:17939-17946). Ten μg of DNA were digested with restriction endonuclease *Eco*RI, electrophoresed in a 1% agarose gel, and blotted onto Nytran membranes according to the manufacturer's protocol. Fifteen μg of total RNA were electrophoresed in a 1.2% formaldehyde-agarose gel and transferred to Nytran membranes. Membranes were hybridized with a probe that contained the IFN-γ coding sequence labeled by random priming (Sambrook et al., 1989, supra) with [$\alpha$-$^{32}$P]dCTP (111 TBq/mmol; 3000 mCi/mmol). Following hybridization, blots were washed in 1 x SSC at 68°C and autoradiographed.

## Results

**Generati n and properties f B16 transfectants.** After co-transfection of plasmids pD5-IFN-γ and pPGKneobpA into B16 melanoma cells, thirty G418-resistant clones were screened for constitutive secretion of IFN-γ into the culture supernatant. Two clones were found to secrete 240 units/ml of IFN-γ after culture of 1 x $10^6$ cells in 2 ml media for two days. A clone designated as B16.IFN-γ was used for the *in vivo* experiments. The production of IFN-γ by this clone remained constant even after prolonged growth *in vitro* in media containing 500 μg/ml antibiotic G418. The *in vitro* growth of the B16.IFN-γ cells was identical to that of B16 parental cells. The expression of class I MHC antigens on parental (Figure 1A) and B16.IFN-γ (Figure 1B) cells was assessed by flow cytometry. B16.IFN-γ cells showed a significant increase in the expression of MHC class I as well as class II antigens compared to the parental cells. Cells co-transfected with plasmids pD5 and pPGKneobpA (B16-pD5neo) were used as a control in the animal experiments. Thus, B16 cells transfected with the pD5 vector only (B16.pD5 cells) exhibited growth comparable to the B16 parental cells.

The expression vector pRc/neoCMV-mB7 was transfected into B16 parental cells and co-transfected into B16.IFN-γ cells along with 1 μg of plasmid pHM24 which contains the hygromycin resistance gene. After selection with antibiotic G418 and hygromycin B, respectively, clones were assayed for expression of B7 molecules by flow cytometry as described in Materials and Methods. The level of B7 expression on parental cells was also determined (Fig. 2A). A clone expressing B7 (B16.B7.7) and a clone co-expressing IFN-γ and B7 (B16.IFN-γ/B7.11) were selected for the *in vivo* experiments (Figs. 2B and 2C, respectively). The expression of B7 molecules also remained constant after prolonged *in vitro* culture in the presence of the appropriate antibiotics.

**Tumorigenic potential of B16.IFN-γ cells in syngeneic C57BL/6J mice.** To examine whether expression of IFN-γ by B16 melanoma cells affects their tumorigenicity, various numbers of B16.IFN-γ and parental cells were injected s.c.

into C57BL/6J mice. B16 parental cells produced a tumor in all the animals injected after latency periods of up to 15 days at doses as low as $1 \times 10^5$ cells. In contrast, $1 \times 10^5$ B16.IFN-$\gamma$ cells produced tumors only in 50% of the animals injected after latency periods of 50 days or more (Fig. 3A). At higher doses ($1 \times 10^6$ cells), all of the mice injected with parental cells had a visible tumor by day 12, whereas the appearance of B16.IFN-$\gamma$ tumors was delayed by almost one month. B16-pD5neo cells showed the same tumorigenic properties as the parental cells (Fig. 3B). These data show that IFN-$\gamma$ secretion can reduce significantly the tumorigenicity of B16 melanoma cells. In addition, we also observed that mice bearing B16.IFN-$\gamma$ tumors were able to survive significantly longer than the control group injected with parental cells (data not shown; Flores, 1994).

**Role of secreted IFN-$\gamma$ on the decreased tumorigenic potential of B16.IFN-$\gamma$ cells.** In order to evaluate the direct role of secreted IFN-$\gamma$ on the observed reduction of the tumorigenicity of transfected B16 cells, the following experiments were performed. First, anti-murine IFN-$\gamma$ polyclonal antiserum was injected i.p. as a single dose of $5 \times 10^4$ neutralization units per mouse, followed by s.c. injection of $1 \times 10^6$ B16.IFN-$\gamma$ cells. Mice injected with non-specific serum or with cells only served as controls. When specific neutralizing antibodies were injected along with the IFN-$\gamma$-secreting cells, tumors appeared significantly faster than the controls. In contrast, non-specific serum did not accelerate the *in vivo* growth of B16.IFN-$\gamma$ cells. The results, shown in Table 1, indicate that secreted IFN-$\gamma$ is responsible for the slower growth of the transfected cells.

**Table 1**

**Effect f in vivo injection of IFN-γ antiserum on the tumorigenicity of B16.IFN-γ cells**

| Serum | Mice with Tumor/Mice Injected |
|---|---|
| Anti-IFN-γ | 8/8 |
| Non-specific | 2/5 |
| None | 2/5 |

C57BL/6J mice were injected s.c. with 1 x $10^6$ B16.IFN-γ cells and i.p. with 5 x $10^4$ neutralization units of a rabbit polyclonal antiserum raised against murine IFN-γ. As controls, one group of mice was injected with non-specific rabbit serum and another group was injected only with cells. Tumor growth was monitored by palpation of the injected site twice a week. The values in the table represent the number of mice with a palpable tumor 30 days after injection (numerator) over the total number of mice injected (denominator).

Second, the tumorigenicity of parental and B16.IFN-γ cells in mice that lack functional IFN-γ receptors (IFN-γR knockout mice) was evaluated. Injection of 1 x $10^6$ B16.IFN-γ cells into IFN-γR knockout mice resulted in development of tumor in all of the injected animals, whereas the same number of cells were completely rejected by wild type 129/SvEv mice. This effect was not due to allorejection since C57BL/6J mice and 129/SvEv mice are both of the H-$2^b$ haplotype. B16 parental cells, in contrast, were equally tumorigenic in both wild type and IFN-γR knockout mice (Table 2).

**Table 2**

**Tumorigenicity of B16 parental and B16.IFN-γ cells in syngeneic mice and in mice with a deletion of the IFN-γ Receptor**

| Cell Inoculum | Number of Cells | Mice with Tumor/Mice Injected | | |
|---|---|---|---|---|
| | | C57BL/6J | 129 SvEv Wild Type | 129/SvEv IFN-γR ko |
| B16 | 1 x $10^6$ | 5/5 | 10/10 | 10/10 |
| B16.IFN-γ | 1 x $10^6$ | 5/5 | 0/10 | 10/10 |

Mice with a deletion of the IFN-γ receptor (IFN-γR knockout, ko), syngeneic 129/SvEv parental mice and C57BL/6J mice were injected with 1 x $10^6$ B16.IFN-γ cells s.c. Tumor appearance was monitored as described. The values in the table represent the number of mice that developed a tumor during an observation period of three months over the number of mice injected.

These results indicate that functional IFN-γ receptors in the host are required for the development of antitumor responses and suggest that secreted IFN-γ plays a direct role on the decreased tumorigenic potential of B16.IFN-γ cells through immunomodulation of the host immune system.

**Histological analysis of B16 parental and B16.IFN-γ tumor tissue.** To determine the immune mechanisms involved in the delayed *in vivo* growth of B16.IFN-γ cells, the histopathology of injection sites or tumor sections at various times after injection was studied. Striking differences were observed in the histological and growth features of the tumors produced by parental and B16.IFN-γ cells (data not shown). B16 tumors showed numerous mitoses (33-36 per five high power fields, HPF; x 400). A few mononuclear inflammatory cells were present in the peritumoral tissues, but virtually no lymphoid cells could be seen within the tumors. This morphology remained essentially the same throughout the observation period except for the

spontaneous necrosis which became more prominent as the tumors grew older. In contrast, the tumors produced by B16.IFN-$\gamma$ cells showed only sparse cells with rare mitoses (3 per five HPF) 12 days after inoculation. Numerous inflammatory cells including lymphocytes, macrophages and neutrophils were present in the peritumoral tissue and within the tumor. A similar pattern was still present on day 22, whereas tumors excised on days 28 and 34 were morphologically similar to the B16 parental controls and displayed a high mitotic activity (30 mitoses per five HPF) and little, if any, lymphocytic infiltration. These observations are supportive of an effect of IFN-$\gamma$ in recruiting effector cells to the injection site associated with a slower growth of the tumor cells.

**Tumorigenicity of B16.IFN-$\gamma$ cells in athymic mice.** Following the observation that a cellular infiltrate, consisting mainly of lymphocytes, was evident in the B16.IFN-$\gamma$ tumors, we assessed the role of T cells in the host response to the injected cells. Athymic mice of C57BL/6J origin and immunocompetent C57BL/6J mice were injected with 1 x $10^6$ B16.IFN-$\gamma$ cells and tumor appearance was monitored as described. Tumors from B16.IFN-$\gamma$ cells developed faster in athymic mice than in control C57BL/6J mice. Twenty-two days after injection, 80% of the athymic mice had a palpable tumor whereas none of the immunocompetent mice exhibited tumor development until day 29 (data not shown). These observations support the notion that T cells are indeed the effector cells that mediate the responses activated by B16.IFN-$\gamma$ cells.

**DNA, RNA and protein analysis of B16.IFN-$\gamma$ tumor tissue.** Histopathological analysis of B16.IFN-$\gamma$ tumor tissue showed that the recruited cellular infiltrate was eventually lost and that this correlated in time with the appearance of tumors. Possibly transfected cells were losing the IFN-$\gamma$ cDNA and, as a consequence, tumors appeared. Analysis of DNA and RNA from tumors resulting from the injection of B16.IFN-$\gamma$ cells failed to detect the transfected DNA or its message. Furthermore, re-cultured tumor cells produced only trace amounts of IFN-$\gamma$ (15-30 units/ml) compared to 240 units/ml secreted by B16.IFN-$\gamma$ cells as determined by interferon

assay of the culture supernatant. These findings support the hypothesis that tumors arose from the outgrowth of cells that lost or have diminished expression of IFN-$\gamma$.

**Tumorigenic potential of B16.B7 or B16.IFN-$\gamma$/B7 cells in syngeneic C57BL/6J mice.** To examine whether expression of the molecule B7 by B16 melanoma cells, alone or in combination with IFN-$\gamma$, affects their tumorigenicity, various numbers of B16.IFN-$\gamma$, B16.B7.7, B16.IFN-$\gamma$/B7.11, and parental cells were injected s.c. into C57BL/6J mice. The tumorigenicity of B16.B7 cells was almost unaffected as compared to that of parental cells (Figure 4). In contrast, cells that expressed increased levels of MHC molecules and B7, and that also secreted considerable amounts of IFN-$\gamma$ into the culture supernatant, were rejected by the majority of the hosts injected. Seven out of 10 mice that were inoculated with 1 x $10^6$ B16.IFN-$\gamma$/B7 cells did not develop a tumor during a six month observation period (Figure 4). Moreover, all (7 out of 7) of the protected animals rejected a tumorigenic dose of parental cells, which indicates that the B16.IFN-$\gamma$/B7 cells were able to induce immune responses that could also recognize the parental tumor (Table 3).

**Table 3**

**Effect of immunization of mice with B16.IFN-$\gamma$/B7 cells on challenge with B16 parental melanoma cells**

| Mice | Mice with Tumor/Mice Injected | % Protection from Challenge |
|---|---|---|
| Naive | 5/5 | 0 |
| Immunized with B16.IFN-$\gamma$/B7 cells | 0/7 | 100 |

C57BL/6J mice were injected s.c. with 1 x $10^6$ B16.IFN$\gamma$/B7 cells. Mice that rejected the tumor cells were challenged on day 62 with 1 x $10^5$ B16 parental cells at the contralateral site. Naive mice injected with 1 x $10^5$ B16 cells served as control. Tumor appearance was monitored as described in Materials and Methods.

A mixture of B16.B7 cells and B16.IFN-$\gamma$ cells were also injected to study whether activation of immune effector mechanisms required that both molecules were co-expressed by the same cell or if the necessary factors could be contributed by different cells. We observed that co-injection of cells that expressed one molecule or the other resulted only in delays in the appearance of tumors which were not different from those observed when B16.IFN-$\gamma$ cells only were injected (Figure 4).

Discussion

This Example shows that secretion of IFN-$\gamma$ by B16 melanoma cells results in a significant reduction in tumorigenicity as compared to parental cells. Most importantly, it demonstrates that tumor cells genetically-modified to express both IFN-$\gamma$ and B7 can induce potent protective antitumor responses. B16 cells secreting IFN-$\gamma$ showed increased surface expression of MHC class I and class II molecules which are required for antigen presentation to both the $CD8^+$ and $CD4^+$ arms of the immune system, respectively. However, the reduced tumorigenicity of B16.IFN-$\gamma$

cells is not only an aftermath of the upregulation in MHC antigen expression since we demonstrated that secreted IFN-$\gamma$ directly affects host effector mechanisms.

The requirement of functional IFN-$\gamma$ receptors for effective rejection of the tumor cells by the recipient mice and the observation that IFN-$\gamma$ antiserum is able to promote B16.IFN-$\gamma$ tumor growth *in vivo* provide evidence for the direct role played by secreted IFN-$\gamma$ on host cells. This effect of IFN-$\gamma$ was unrelated to its well known antiproliferative activity since B16.IFN-$\gamma$ cells had an *in vitro* growth rate similar to that of parental cells. In addition, injection of B16.IFN-$\gamma$ cells into C57BL/6J athymic mice resulted in the acceleration of tumor growth. These observations indicate that T cells are primarily responsible for the reduced tumorigenicity of B16.IFN-$\gamma$ cells. Further evidence for the involvement of T cells in the observed effects comes from the histological analysis of the injection sites. A massive infiltrate consisting of lymphocytes and granulocytes was evident in mice inoculated with B16.IFN-$\gamma$ cells, which was absent in the parental tumors. The eventual appearance of B16.IFN-$\gamma$ tumors correlated in time with the loss of this cellular infiltrate, suggesting that the tumor cells had possibly lost expression of IFN-$\gamma$. This hypothesis was substantiated by the observation that IFN-$\gamma$ tumors had little expression of IFN-$\gamma$. Thus, it is likely that tumors arose from cells that had lost the ability to produce IFN-$\gamma$. Taken together, these observations indicate that secreted IFN-$\gamma$ plays a direct immunostimulatory role that results in the decreased tumorigenic potential of B16.IFN-$\gamma$ cells.

These results also raise a word of caution for future gene therapy studies. The loss of cytokine production during *in vivo* growth has been reported (Hock et al., 1993, Proc. Natl. Acad. Sci. USA 90:2774-2778; Tepper et al., 1989, Cell 57:503-512; Esumi et al., 1993, Cancer Res. 51:1185-1189; Aoki et al., 1992, Proc. Natl. Acad. Sci. USA 89:3850-38554. ) and represents a challenge to overcome in the use of gene-modified cells in human therapy. The loss of the transfected gene might be prevented or reduced with the use of retroviral vectors (Mulligan, 1993; Dougherty and Ron, 1994), which represent the vector of choice for human gene therapy not

only because they have higher gene transfer efficiencies but also because they provide for stable integration of the DNA and potential for multiple insertions at different sites.

IFN-γ is also known as immune interferon for its role in the activation and regulation of immune effector mechanisms such as cytotoxic T lymphocytes (CTL), NK cells and macrophages (Trinchieri and Perussia, 1985, Immunol. Today 6:131-136; Pestka et al., 1987, Biochem. 56:727-777), and of the expression of tumor associated antigens and molecules required for the presentation of antigenic determinants to CTL and helper T cells such as adhesion and MHC molecules (Dustin et al., 1986, J. Immunol. 137(1):245-254; Altmann et al., 1989, Nature 338:512-514; Guadagni et al., 1989, J. Natl. Cancer Inst. 81:502-512. ; Mortarini et al., 1990, Int. J. Cancer 45:334-341. ). IFN-γ-gene transfection into various tumor models has resulted in a decrease in their tumorigenicity that was correlated by some investigators with upregulation of surface MHC molecules (Gansbacher *et al.*, 1990 supra; Restifo *et al.*, 1992, supra; Porgador *et al.*, 1993, supra), whereas others have presented evidence showing that increased levels of MHC expression were not sufficient to elicit effective antitumor immune responses (Watanabe *et al.*, 1989, supra; Esumi *et al.*, 1993, supra; Lollini *et al.*, 1993, supra). It appears that, in addition to the upregulation of surface molecules, IFN-γ activates other mechanisms that result in the development of potent immune responses. This study helps to define the role of secreted IFN-γ in the observed reduced tumorigenicity of the transfectants. With the aid of the IFN-γR knockout mice we have proved that IFN-γ activates immune effector cells via interactions with specific receptors on host cells. This observation was supported by histological examination that showed the presence of a cellular infiltrate at the injection site and by the effects of IFN-γ antibodies in blocking the observed responses.

The studies performed with B16.B7 cells suggest that, at least in the B16 melanoma model, B7 expression alone is insufficient for reducing the ability of the tumor cells to grow *in vivo*. These observations contrast with what has been reported with other

tumor models (Chen et al., 1992, Cell 71:1093-1102; Baskar et al., 1993, Proc. Natl. Acad. Sci. USA 90:5687-5690. ; Townsend and Allison, 1993, Science 259: 68-370. ); perhaps the low expression of MHC molecules on B16 melanoma cells contributes to the lack of immunogenicity of B16.B7 cells. This hypothesis is supported by the observation that B16.IFN-$\gamma$/B7 cells can provide all the elements necessary for effective activation of immune responses: MHC molecules that can present tumor epitopes to the T cell receptor, the B7 molecule which provides the costimulatory signal that is crucial for T cell activation, and IFN-$\gamma$ which plays a role in the stimulation of specific T effector cells (Chen et al., 1986, Eur. J. Immunol. 16:767-770. ; Maraskovsky et al., 1989, J. Immunol. 143:1210-1214). Results from an additional experiment in which a mixture of B16.B7 cells and B16.IFN-$\gamma$ cells were injected indicated that MHC and B7 molecules must be expressed on the same cell since co-injection of cells that expressed one molecule or the other resulted only in delays in the appearance of tumors.

In conclusion, these data demonstrate that tumor cells modified to express IFN-$\gamma$ and B7 can be used effectively to generate specific immune responses against parental cells. This effect of IFN-$\gamma$ is probably a result of both the augmentation of MHC antigen expression and the direct activation of T effector cell mechanisms to recognize neoplastic cells. B7, on the other hand, plays a more defined role in the activation of T effector cells. Expression of this molecule along with MHC antigens may give tumor cells the ability to present tumor specific epitopes appropriately to the effector cells of the immune system. These studies show that tumor cells can be made efficient antigen presenting cells by modifying them genetically to express two defined molecules. The use of tumor cells with antigen presenting and immunostimulatory capabilities as antitumor vaccines has great potential in human cancer therapy, specifically as an adjunct to conventional therapies.

EXAMPLE 2: Gene Therapy of Melanoma With Interferon $\alpha$

In this Example, the highly aggressive murine B16 melanoma was engineered to secrete IFN-$\alpha$ constitutively. Cells expressing IFN-$\alpha$ were injected into syngeneic C57BL/6 mice and the mice were monitored for tumor development. Secretion of IFN-$\alpha$ by B16 melanoma cells completely abrogated their tumorigenicity in syngeneic mice. IFN-$\alpha$-secreting cells also abrogated the tumorigenicity of IFN-$\gamma$-secreting and TNF-$\alpha$-secreting cells when injected in combination whereas cells secreting either IFN-$\gamma$ or TNF-$\alpha$ grow progressively in mice when injected alone. Moreover, protected animals developed significant immunity against subsequent challenge with parental cells. Injection of parental cells and IFN-$\alpha$-secreting cells together in a mixed tumor transplantation assay resulted in a significant reduction of tumorigenicity of the parental cells. Histopathological studies of the tissues from the injection site of the mice inoculated with a combination of parental and B16.IFN-$\alpha$ cells revealed the existence of a massive cellular infiltrate composed of lymphocytes and granulocytes at an early stage (7-11 days). In the later stages (22 days), no recognizable tumor tissue was detected. Injection of irradiated IFN-$\alpha$-secreting cells in the mice carrying an established tumor completely prevented tumor development in 80% of the treated mice when injection was performed on the same side as the tumors. Injection of irradiated IFN-$\alpha$-secreting cells in the contralateral site showed much less effect on the established tumor. Systemic antitumor effects on the established tumor can be enhanced by using a combination of irradiated IFN-$\alpha$ and IFN-$\gamma$ secreting cells as a vaccinating inoculum.

## Materials and Methods

**Mice and Cell Lines.** The mice and BL6 cell lines are described in Example 1, *supra*.

**Expression vectors for transfection.** A 680 bp *Hae*III/*Dra*I fragment containing the complete coding sequence of murine IFN-$\alpha$A from the plasmid pBD1 (Daugherty B. et al., 1984, J. Interferon Res., 4:635-643) was subcloned into the *Hpa*I site of the

retroviral expression vector pLNCX (Miller AD and Rosman GJ, 1989, Biotechniques 7(9):980-990), downstream from the human cytomegalovirus (CMV) promoter. The pLNCX-IFN-$\alpha$ construct, was used for transfection into B16 cells. This plasmid contains the bacterial neomycin resistance gene downstream of the retroviral LTR.

The pD5-IFN-$\gamma$ construct described in Example 1 was used for transfection into B16 cells along with plasmid pPGKneobpA.

An *Eco*RI/*Hind*III fragment containing the mouse TNF-$\alpha$ coding sequence and part of the 3′ untranslated region from the plasmid pGEM-MTNF was made and the 5′ overhang generated by *Eco*RI digestion was filled in with the Klenow fragment of the DNA polymerase I. This fragment was subcloned into the retroviral expression vector pLNCX (Miller AD and Rosman GJ, 1989, Biotechniques 7(9):980-990) which was cut with the restriction endonuclease *Hind*III and *Cla*I (5′ overhang of *Cla*I site of the vector was filled in with the Klenow fragment of the DNA polymerase I) sites downstream of the human cytomegalovirus (CMV) promoter. This plasmid also contains the bacterial neomycin resistance gene downstream of the retroviral long terminal repeat (LTR). The resulting plasmid pLNCX-TNF-$\alpha$ was used for transfection.

**DNA transfection.** Twenty $\mu$g of the plasmid pLNCX-IFN-$\alpha$ was transfected into $3.5 \times 10^5$ B16 cells by the calcium phosphate coprecipitation method (Chen C and Okayama H, 1988, Mol. and Cell. Biol. 7:2745-2752) and selected in a medium containing 1 mg/mL of antibiotic G418 (Geneticin; Gibco). Plasmids pD5-IFN-$\gamma$ and pPGKneobpA were tranfected into B16 cells as described in Example 1. The pLNCX-TNF-$\alpha$ plasmid was introduced into B16 cells via retroviral gene transduction (Markowizt et al., 1988, Virology, 167:400-406). Antibiotic G418 resistant colonies were isolated and screened for the expression of the particular cytokine transfected.

**Interferon assay.** G418-resistant clones were assayed for the production of IFN-$\alpha$ by the cytopathic effect inhibition assay with encephalomyocarditis virus (EMCV) on mouse L929 cells (Fammilletti et al., 1981, Academic Press. **78**:430-435). G418-resistant clones were seeded in 6-well tissue culture plates at 1 x $10^6$ cells per well in 2 mL DMEM containing 10% FBS without antibiotic G418. After 24 or 48 hours, culture supernatants were serially diluted 2-fold in 96-well microtiter plates and 4 x $10^4$ mouse L929 cells were added per well. Following overnight incubation at 37°C, EMCV was added and the incubation continued for an additional 24 hours after which plates were washed and then stained with crystal violet to observe residual viable cells. IFN activity is expressed in units/mL calibrated against the NIH reference standard for mouse IFN-$\alpha$/ß G-002-904-511. IFN-$\gamma$ production of the clones transfected with pD5-IFN-$\gamma$ was assayed as described above and the activity was calibrated against NIH reference standard Gg02-901-533 for mouse IFN-$\gamma$.

**TNF assay.** Culture supernatants from the G418 resistant clones were assayed following the method of Asher *et al.*(1987, J. Immunol. (38:963-974). Briefly, 1 x $10^6$ cells were seeded in a 6-well dish in 2 mL of media without any G418. Supernatants were collected after 24 hrs and serially diluted 2-fold in 96-well microtiter plates. L929 cells (4 x $10^4$) were added in the presence of actinomycin D (1 $\mu$g/mL). Following overnight incubation at $37^0$ C, plates were stained with crystal violet to determine the endpoint of growth inhibition. TNF-$\alpha$ activity was expressed in units/mL against the NIH reference standard for recombinant human TNF-$\alpha$.

**Flow cytometry.** Unmodified B16 cells and B16 cells expressing mouse IFN-$\alpha$ (B16.IFN-$\alpha$), IFN-$\gamma$ (B16.IFN-$\gamma$), TNF-$\alpha$ (B16.TNF-$\alpha$) were incubated with monoclonal antibodies 28.8.6 (anti-H-2K$^b$) (Ozato K and Sachs DH, 1981, J. Immunol. 126:317-321), or AF6-120.1.2 (anti-I-A$^b$) from American Type Culture **Collection** (HB163) as described in Example 1, *supra*.

**Cell gr wth.** To assess whether cytokine production affects the growth of cytokine-secreting cells *in vitro* growth assays were performed . B16 or cytokine-secreting

cells were seeded at $1 \times 10^5$ cells per well in 6-well tissue culture plates and incubated at 37°C. After 48 hours cells were counted after staining with trypan blue to exclude dead cells.

**Tumor growth.** C57BL/6 male mice were injected subcutaneously (s.c.) on the flank with various numbers of cells in 0.2 mL of Hank's balanced saline solution (HBSS). Tumor growth was followed and recorded as described in Example 1, *supra*. As in Example 1, mice that rejected the primary injection of tumor cells were challenged s.c. with parental B16 cells on the opposite flank and animals with massive tumor burden were sacrificed.

**Histological analysis of tumor tissue.** Tissue at the site of injection was obtained at various times and fixed in 10% phosphate-buffered formalin as described in Example 1, *supra*.

**Immunotherapy of established tumor.** Mice were injected with $1 \times 10^5$ B16 cells and three days later they were treated by injection with 4-5 $\times 10^6$ irradiated (10,000 rads) B16 parental or cytokine secreting cells once a week for three consecutive weeks at the contralateral site. In a separate experiment mice were injected with $5 \times 10^6$ irradiated B16.IFN-$\alpha$ cells at the tumor site 3 or 7 days after the injection of $1 \times 10^5$ parental tumor cells. This treatment was repeated twice at weekly intervals.

## Results

**Generation and properties of B16 clones expressing different cytokines.** B16 melanoma cells were transfected with the plasmid pLNCX-IFN-$\alpha$ by calcium phosphate coprecipitation method (Chen, 1988, *supra*). Antibiotic G418-resistant clones were screened for the constitutive secretion of IFN-$\alpha$. A clone secreting IFN-$\alpha$ at 30,000 units/mL/24hrs/$10^6$ cells, designated as B16.IFN-$\alpha$, was used for the *in vivo* experiments. Cells that were transfected with the plasmid pLNCX were used as a control in the animal experiments. Cells transfected with pD5IFN-$\gamma$ and pPGKneobpA were screened for the secretion of IFN-$\gamma$ and a clone secreting IFN-$\gamma$

at 240 units/mL/48 hr/$10^6$ cells designated as B16.IFN-$\gamma$ was selected for further studies. B16 clones generated by transduction of pLNCX-TNF-$\alpha$ plasmid were assayed for TNF-$\alpha$ secretion. A clone secreting TNF-$\alpha$ at 1500 units/mL/24 hr/$10^6$ cells designated as B16.TNF-$\alpha$ were selected for injection.

The expression of MHC antigens in B16 parental and cytokine-secreting cells was assessed by cytofluorographic analysis. B16.IFN-$\alpha$ cells expressed significantly higher MHC class I antigens but not MHC class II antigens as compared to the parental cells. B16.IFN-$\gamma$ cells were significantly higher in both MHC class I and class II antigens whereas B16.TNF-$\alpha$ cells did not show any appreciable increase of MHC antigen expression as compared to the parental cells (data not shown).

Since IFN-$\alpha$ is known to have growth inhibitory effect on a variety of cell lines, secretion of IFN-$\alpha$ by B16 cells on their *in vitro* growth rate was tested. B16 parental and B16.IFN-$\alpha$ cells were plated at a concentration of 1 x $10^5$ cells per well in 6- well plates and the live cells were counted after 48 hours by trypan blue exclusion. B16.IFN-$\alpha$ cells grew at one third the rate of B16 parental cells. Addition of up to 10,000 units/mL of IFN-$\alpha$ to B16 cells exogenously, however, did not affect the growth of B16 cells. B16.IFN-$\gamma$ and B16.TNF-$\alpha$ cells did not show any significant change in their growth rate as compared to the parental cells.

**Complete abrogation of the tumorigenicity of B16 cells secreting IFN-$\alpha$.** Although the immunomodulatory effects of IFN-$\alpha$ are less potent than those of IFN-$\gamma$, constitutive secretion of IFN-$\alpha$ by tumor cells at the tumor site may augment host immune responses against a tumor, especially in some hematological malignancies that respond to the systemic administration of IFN-$\alpha$ (Ogasawara M and Rosenberg SA, 1993, Cancer Res. 53:3561-3568). To examine whether the constitutive secretion of IFN-$\alpha$ by B16 melanoma cells reduces their tumorigenicity *in vivo*, B16.IFN-$\alpha$ and B16 parental cells were injected into syngeneic C57BL/6 mice. All mice injected with B16 parental cells developed tumors, which started to appear at

day 7, whereas none of the mice injected with B16.IFN-α cells ever developed a tumor during an observation period of over 2 months (Figure 5).

**Tumorigenicity of a combination of B16.IFN-α and B16.IFN-γ or B16.IFN-α and B16.TNF-α cells.** Since IFN-α and IFN-γ are highly synergistic (Fleishmann, 1986 *supra*) we injected a combination of B16.IFN-α and B16.IFN-γ cells into syngeneic C57BL/6 mice. Groups of mice injected with either B16.IFN-α or B16.IFN-γ cells served as controls. Eighty percent of the mice injected with B16.IFN-γ cells developed tumor with a latency period of 20 days while only 5% of the mice injected with a combination of B16.IFN-α and B16.IFN-γ cells developed tumor with a latency period of 50 days. Injection of B16.IFN-α and B16.IFN-γ cells on the contralateral sites did not result in a significant effect on the growth of B16.IFN-γ cells (data not shown). None of the mice injected with B16.IFN-α cells developed tumor during the 80 days observation period (Figure 6).

Balkwill *et al.* (1986, *supra*) showed that a combination of TNF-α with either IFN-α or IFN-γ resulted in an antitumor effect greater than either cytokine alone, although the effects were more marked with a combination of IFN-γ and TNF-α than with IFN-α and TNF-α. Based on this observation, it remained uncertain whether a combination of B16.IFN-α and B16.TNF-α cells can induce the rejection of cytokine-secreting cells when injected in syngeneic mice. B16.TNF-α cells developed tumors in mice, with 100% of the mice exhibiting tumors within 23 days, whereas none of the mice injected with a combination of B16.IFN-α and B16.TNF-α cells developed tumor during a 65 days observation period. Injection of B16.IFN-α and B16.TNF-α at contralateral sites however, generated a smaller, but nevertheless significant effect on the growth of B16.TNF-α cells (Figure 7).

**Generation of long term protective immunity.** Both IFN-α and IFN-γ exert multiple antitumor effects that may be categorized either into the direct antiproliferative activity against the tumor or into activity indirectly mediated through the host immune system, including the enhancement of MHC class I and class II

antigen expression, activation of macrophages and natural killer cells, generation of cytotoxic T lymphocytes, and induction of tumor associated antigens (Balkwill FR, 1989, "Cytokines in Tumor Therapy, *In Interferons*, Oxford University Press: New York, pp. 8-57). To examine whether the antitumor effects of IFN-$\alpha$ were due to a direct antiproliferative effect or involved host immune responses, mice that rejected the cytokine-secreting cells were challenged with $1 \times 10^5$ B16 cells on the contralateral sites. Naive mice injected with $1 \times 10^5$ B16 cells served as controls. Mice immunized with B16.IFN-$\alpha$ cells showed 40% protection while mice immunized with a combination of B16.IFN-$\alpha$ and B16.IFN-$\gamma$ or B16.IFN-$\alpha$ and B16.TNF-$\alpha$ showed 60%-67% and 80% protective immunity, respectively, against subsequent challenge with parental cells. Results are shown in Table 4.

**Table 4. Generation of protective immunity against subsequent challenge.**

| Immunogen | Cells injected | Day of challenge | Mice with tumor/Mice injected | % Protected |
|---|---|---|---|---|
| B16.IFN-$\alpha$ | $1 \times 10^6$ | 65 | 6/10 | 40% |
| B16.IFN-$\alpha$ | $1 \times 10^6$ | 71 | 3/5 | 40% |
| B16.IFN-$\alpha$<br>B16.IFN-$\gamma$ | $5 \times 10^5$<br>$5 \times 10^5$ | 65 | 4/10 | 60% |
| B16.IFN-$\alpha$<br>B16.IFN-$\gamma$ | $1 \times 10^6$<br>$1 \times 10^6$ | 77 | 1/3 | 67% |
| B16.IFN-$\alpha$<br>B16.TNF-$\alpha$ | $1 \times 10^6$<br>$1 \times 10^6$ | 65 | 2/10 | 80% |

Mice were injected with live cytokine-secreting cells and challenged with $1 \times 10^5$ B16 parental cells at the contralateral site as described in the text.

**Inhibition of growth of B16 cells in a mixed tumor transplantation assay.** The ability of B16.IFN-$\alpha$ cells to inhibit the growth of B16 parental cells in a mixed

tumor transplantation assay was assessed. Mice were injected with a combination of B16.IFN-$\alpha$ and B16 parental cells. In another group of mice B16 parental and B16.IFN-$\alpha$ cells were injected contralaterally and the tumor appearance in the two groups was compared. Seventy percent of the mice injected with a mixture of B16 and B16.IFN-$\alpha$ cells remained tumor-free over a three month observation period (Figure 8). Mice injected with B16.IFN-$\alpha$ and B16 cells on the contralateral sites showed only a slight delay in the growth of parental cells (data not shown).

**Recruitment of immune effector cells to the injection site.** Histopathological examination of the samples taken from the injection site of mice inoculated with B16.IFN-$\alpha$ cells revealed a totally necrotic tumor nodule with a prominent peri- and intra-tumoral infiltrate composed of lymphocytes and granulocytes on days 7 and 14. Biopsies taken at day 21 from the injection site revealed no tumor. Tumor samples taken from mice injected with B16 parental cells did not show appreciable cellular infiltrate at any stages. When a mixture of B16.IFN-$\alpha$ and B16.IFN-$\gamma$ were injected, a totally necrotic tumor nodule with a marked cellular infiltrate composed of lymphocytes and granulocytes was visible on days 7 and 14, while samples taken on day 21 failed to show any tumor at all. When B16.IFN-$\alpha$ and B16.IFN-$\gamma$ cells were injected on contralateral sides, on both sides necrotic tumor nodules with massive cellular infiltrate comprising both lymphocytes and granulocytes were visible on days 7 and 14. Samples taken from the mice injected with a combination of B16.IFN-$\alpha$ and B16.TNF-$\alpha$ cells revealed the existence of prominent necrosis and a marked infiltrate consisting of lymphocytes and granulocytes on day 7 and 14. On day 21 prominent granulocytic and lymphocytic infiltrate was visible but no tumor cells were detectable. Massive cellular infiltrate composed of lymphocytes and granulocytes were evident at an early stage (7-11 days) when a combination of B16 parental and B16.IFN-$\alpha$ cells were injected whereas in later stages (day 22) no recognizable tumor was detected. The existence of extensive cellular infiltrates including lymphoid cells indicates the involvement of host immune responses rather than only direct antiproliferative effects of these cytokines on the tumor cells.

**Gr wth f B16.IFN-$\alpha$ cells in immunoc mpr mised mice.** To determine whether the abrogation of tumorigenicity of B16 cells by IFN-$\alpha$ is due to the direct antiproliferative effect of this cytokine and whether the host T cells play any role in the effect observed, we injected 1 x $10^6$ B16.IFN-$\alpha$ or B16 parental cells into athymic mice (nu/nu). C57BL/6 mice injected with parental and B16.IFN-$\alpha$ cells served as control. During a two month observation period athymic mice injected with B16.IFN-$\alpha$ cells failed to develop tumor while all athymic mice injected with B16 parental cells developed tumors within one week (data not shown). Inability of B16.IFN-$\alpha$ cells to grow in both immunocompetent and T cell deficient mice suggests several possibilities. In part, failure of B16.IFN-$\alpha$ cells *in vivo* may be related to the direct antiproliferative properties of IFN-$\alpha$. Moreover, rejection of B16.IFN-$\alpha$ cells may not be mediated by T cells, but rather by NK cells or macrophages. Furthermore, it is conceivable that B16.IFN-$\alpha$ cells completely lost their tumorigenic potential *in vivo*.

This last possibility prompted an investigation the consequence of injection of a 10-fold higher number of cells in C57BL/6 mice. Injection of 1 x $10^7$ B16.IFN-$\alpha$ cells led to tumor development in all 5 mice inoculated, but the tumor receded in one animal and stopped growing in the other 4 mice after some time.

**Local and systemic curative potential of B16.IFN-$\alpha$ cells.** To evaluate whether the injection of irradiated B16.IFN-$\alpha$ cells can affect the growth of an established tumor, the following experiments were performed. Mice injected with 1 x $10^5$ B16 cells were treated with three injections of 5 x $10^6$ irradiated B16.IFN-$\alpha$ cells in the tumor challenge area on days 4, 11 and 18. Tumor growth in the treated mice was compared with that in the control group, which received no further treatment after the initial injection of 1 x $10^5$ tumor cells. The tumor growth was impaired significantly in mice bearing a 3-day old tumor when treated with three injections of irradiated IFN-$\alpha$ secreting cells at weekly intervals. None of the treated mice developed a tumor for over two months and only 20% of the mice developed tumors after 70 days, while all untreated mice developed tumors within 35 days (Figure 9). Although

this treatment was less effective on a 7-day old tumor, a significant difference in tumor growth between treated and untreated mice was observed (Figure 10).

To examine the systemic curative potential of irradiated B16.IFN-α cells, mice bearing 3-day old tumors were injected contralaterally with 4-5 x $10^6$ irradiated B16.IFN-α cells three times at weekly interval. A control group was treated with irradiated B16 parental cells. Tumor growth was blocked in 26% of the mice treated with B16.IFN-α cells and in only 14% of the mice that received irradiated parental cells. However, this difference was not statistically significant. The tumor was cured in 35% of the mice when treated with a combination of irradiated B16.IFN-α and B16.IFN-γ cells. This treatment conferred significant protection compared to the treatment with parental cells only ($P < 0.05$). Cumulative data from 4 different experiments are shown in Table 5.

**Table 5.** Treatment of mice bearing tumor with irradiated parental and cytokine secreting cells.

| Immunogen | Mice with tumor/ mice injected | % mice with tumor | % mice protected |
|---|---|---|---|
| None | 22/26 | 85% | 15% |
| B16 | 18/21 | 86% | 14% |
| B16.IFN-α | 17/23 | 74% | 26% (*P*=.15) |
| B16.IFN-γ | 27/29 | 93% | 7% (*P*=.21) |
| B16.IFN-α B16.IFN-γ | 15/23 | 65% | 35% (*P*<.05) |

Mice were injected with 1 x $10^5$ B16 parental cells s.c. on the flank. Three days later they were injected with 4-5 x $10^6$ irradiated cells on the opposite flank and this treatment was repeated twice at weekly interval. Cumulative data from 4 different experiments are shown.

## Discussion

IFN-$\alpha$ has several important antitumor properties ranging from growth arrest to the induction of immunologically important molecules such as MHC class I antigens and gp96 (Gutterman, 1994, Proc. Natl. Acad. Sci. USA, 91:1198-1205), (Pestka S. et al.,1987, Ann. Rev. Biochem. 56:727-777), (Lengyl, 1993, Proc. Natl. Acad. Sci. USA 90:5893-5895), (Srivastava, 1993, Adv. Cancer Res. 62:153-177). Systemic administration of IFN-$\alpha$ is effective in the treatment of several hematological malignancies while the result of IFN-$\alpha$ therapy of solid tumors does not appear encouraging due to the toxicity associated with high doses of IFN-$\alpha$ (Gutterman, 1994, *supra*). Our study was designed to test the efficacy of IFN-$\alpha$ in gene therapy of B16 melanoma. This Example shows, for the first time, that secretion of IFN-$\alpha$ by B16 melanoma cells completely abrogated their tumorigenicity. Thus, introduction of expression vectors for IFN-$\alpha$ into cells can be used to avoid the need for irradiation. B16.IFN-$\alpha$ cells can also prevent the growth of B16.IFN-$\gamma$ and B16.TNF-$\alpha$ cells when injected in combination with either cells.

Mice that rejected the cytokine-secreting cells showed significant protective immunity against subsequent challenge with parental cells. Treatment of tumor-bearing mice with irradiated B16.IFN-$\alpha$ cells locally caused regression of tumor in 80% of the mice, although injection of B16.IFN-$\alpha$ cells on the contralateral site showed much less effect on the tumor growth. This effect on tumors can be enhanced by the use of a combination of irradiated B16.IFN-$\alpha$ and B16.IFN-$\gamma$ cells.

It is likely that the therapeutic effect seen upon the injection of irradiated B16.IFN-$\alpha$ at the tumor site is due both to the direct antiproliferative effect of IFN-$\alpha$ and to the induction of some host immune responses. These conclusions are consistent with the histological findings. Histopathological studies of tumor samples taken from the mice injected with a combination of B16 and B16.IFN-$\alpha$ cells revealed the existence of extensive lymphocytic and granulocytic infiltrate, whereas injection of B16 cells alone did not reveal any appreciable infiltrate. These data suggest that secreted IFN-$\alpha$ elicited a local immune response which contributed to the rejection of tumor cells.

Angiogenesis is a crucial step in tumor development. The number of microvessels in invasive breast carcinoma and other tumors predicts the likelihood of metastatic progression (Folkman J and Shing Y, 1992, J. Biol. Chem. 267:10931-10934). IFN-$\alpha$ is known to have antiangiogenic activity. The therapeutic effects seen in the regression of 3-day old tumors upon injection of irradiated B16.IFN-$\alpha$ cells at the local tumor site could be due also in part to the impairment of blood vessel development by IFN-$\alpha$.

The interferons, like most other cytokines, are produced by the body to act in an autocrine and paracrine fashion. When used as a systemic therapy, certain toxic effects are seen. This is the first report of the use of IFN-$\alpha$ in a gene therapy model with a solid tumor. Comparative study of different cytokines in MBT and B16.F10 cells reported by Saito *et al.* (1994, Cancer Res. 54:3516-20) and Dranoff *et al.*(1993, Proc. Natl. Acad. Sci. U.S.A. 90:3539-43) did not include this important cytokine. Although at this point the mechanism by which IFN-$\alpha$ exerts its antitumor effect remains unclear, our results demonstrate that the use of irradiated B16.IFN-$\alpha$ cells as a tumor vaccine can effectively inhibit the tumor growth when used locally. The results of this Example demonstrate that it may be possible to eliminate an established tumor by optimization of dosages and combination of cytokines.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art to which the invention pertains from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein. The disclosures of which are incorporated herein by reference in their entireties.

WHAT IS CLAIMED IS:

1. A solid tumor vaccine comprising tumor cells transfected to express interferon-$\alpha$ in a pharmaceutically acceptable excipient.

2. The solid tumor vaccine of claim 1, wherein the tumor cells are also transfected to express an immunomodulatory molecule, with the proviso that the immunomodulatory molecule is not interferon-$\alpha$.

3. The solid tumor vaccine of claim 2, wherein the immunomodulatory molecule is selected from the group consisting of interferon-$\gamma$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, B7-1 T cell costimulatory molecule, B7-2 T cell costimulatory molecule, immune cell adhesion molecule (ICAM) -1 T cell costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

4. The solid tumor vaccine of claim 1, further comprising additional tumor cells transfected to express an immunomodulatory molecule, with the proviso that the immunomodulatory molecule is not interferon-$\alpha$.

5. The solid tumor vaccine of claim 4, wherein the immunomodulatory molecule is selected from the group consisting of interferon-$\gamma$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, B7-1 T cell costimulatory molecule, B7-2 T cell costimulatory molecule, immune cell adhesion molecule (ICAM) -1 T cell costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

6. The solid tumor vaccine of claim 1 further comprising a soluble immunomodulatory molecule.

7. The solid tumor vaccine of claim 6, wherein the soluble immunomodulatory molecule is selected from the group consisting of interferon-$\gamma$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

8. A method for treating a solid tumor comprising introducing into a subject suffering a solid tumor a therapeutically effective number of tumor cells from a solid tumor, which tumor cells are transfected to express interferon-$\alpha$.

9. The method according to claim 8, wherein the tumor cells are also transfected to express an immunomodulatory molecule, with the proviso that the immunomodulatory molecule is not interferon-$\alpha$.

10. The solid tumor vaccine of claim 9, wherein the immunomodulatory molecule is selected from the group consisting of interferon-$\gamma$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, B7-1 T cell costimulatory molecule, B7-2 T cell costimulatory molecule, immune cell adhesion molecule (ICAM) -1 T cell costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

11. The method according to claim 8, further comprising introducing a therapeutically effective number of additional tumor cells transfected to express an immunomodulatory molecule into the subject, with the proviso that the immunomodulatory molecule is not interferon-$\alpha$.

12. The solid tumor vaccine of claim 11, wherein the immunomodulatory molecule is selected from the group consisting of interferon-$\gamma$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2,

interleukin-7, interleukin-12, interleukin-15, B7-1 T cell costimulatory molecule, B7-2 T cell costimulatory molecule, immune cell adhesion molecule (ICAM) -1 T cell costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

13. The method according to claim 8 further comprising introducing an therapeutically effective amount of a soluble immunomodulatory molecule into the subject.

14. The method according to claim 13, wherein the soluble immunomodulatory molecule is selected from the group consisting of interferon-$\gamma$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

15. The method according to claim 8, wherein the tumor cells are from the tumor in the subject.

16. The method according to claim 8, wherein the tumor cells are introduced in proximity of the tumor in the subject.

17. A method for treating a solid tumor comprising introducing into a subject suffering from a solid tumor an expression vector directed to cells of the solid tumor, which expression vector codes on expression for interferon-$\alpha$.

18. The method according to claim 17, further comprising introducing into the subject a second expression vector directed to cells of the solid tumor, which second expression vector codes on expression for an immunomodulatory molecule.

19. The method according to claim 17, wherein the expression vector directed to cells of the solid tumor additionally codes on expression for an immunomodulatory molecule.

20. A solid tumor vaccine comprising tumor cells transfected to express interferon-$\gamma$ and an immunomodulatory molecule, in a pharmaceutically acceptable excipient.

21. The solid tumor vaccine of claim 20, wherein the immunomodulatory molecule is selected from the group consisting of interferon-$\alpha$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, B7-1 T cell costimulatory molecule, B7-2 T cell costimulatory molecule, immune cell adhesion molecule (ICAM) -1 T cell costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

22. The solid tumor vaccine of claim 20, further comprising additional tumor cells transfected to express an immunomodulatory molecule, with the proviso that the immunomodulatory molecule is not interferon-$\gamma$.

23. The solid tumor vaccine of claim 22, wherein the immunomodulatory molecule is selected from the group consisting of interferon-$\alpha$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, B7-1 T cell costimulatory molecule, B7-2 T cell costimulatory molecule, immune cell adhesion molecule (ICAM) -1 T cell costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

24. The solid tumor vaccine of claim 20 further comprising a soluble immunomodulatory molecule.

25. The solid tumor vaccine of claim 24, wherein the soluble immunomodulatory molecule is selected from the group consisting of interferon-$\alpha$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

26. A method for treating a solid tumor comprising introducing into a subject suffering the solid tumor a therapeutically effective number of tumor cells from a solid tumor, which tumor cells are transfected to express interferon-$\gamma$ and an immunomodulatory molecule, with the proviso that the immunomodulatory molecule is not interferon-$\gamma$.

27. The solid tumor vaccine of claim 26, wherein the immunomodulatory molecule is selected from the group consisting of interferon-$\alpha$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, B7-1 T cell costimulatory molecule, B7-2 T cell costimulatory molecule, immune cell adhesion molecule (ICAM) -1 T cell costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

28. The method according to claim 26, further comprising introducing a therapeutically effective number of additional tumor cells transfected to express an immunomodulatory molecule into the subject, with the proviso that the immunomodulatory molecule is not interferon-$\gamma$.

29. The solid tumor vaccine of claim 28, wherein the immunomodulatory molecule is selected from the group consisting of interferon-$\alpha$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, B7-1 T cell costimulatory molecule, B7-2 T cell costimulatory molecule, immune cell adhesion molecule (ICAM) -1 T cell

costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

30. The method according to claim 26 further comprising introducing an therapeutically effective amount of a soluble immunomodulatory molecule into the subject.

31. The solid tumor vaccine of claim 30, wherein the soluble immunomodulatory molecule is selected from the group consisting of interferon-$\alpha$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

32. The method according to claim 26, wherein the tumor cells are from the tumor in the subject.

33. The method according to claim 26, wherein the tumor cells are introduced in proximity of the tumor in the subject.

34. A method for treating a solid tumor comprising introducing into a subject suffering from a solid tumor an expression vector directed to cells of the solid tumor, which expression vector codes on expression for interferon-$\gamma$, and introducing into the subject a second expression vector directed to cells of the solid tumor, which second expression vector codes on expression for an immunomodulatory molecule, with the proviso that the immunomodulatory molecule is not interferon-$\gamma$.

35. The method according to claim 34, wherein the immunomodulatory molecule is selected from the group consisting of interferon-$\alpha$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, B7-1 T cell costimulatory molecule, B7-2

T cell costimulatory molecule, immune cell adhesion molecule (ICAM) -1 T cell costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

36. A method for treating a solid tumor comprising introducing into a subject suffering from a solid tumor an expression vector directed to cells of the solid tumor, which expression vector codes on expression for interferon-$\gamma$ and an immunomodulatory molecule, with the proviso that the immunomodulatory molecule is not interferon-$\gamma$.

37. The method according to claim 36, wherein the immunomodulatory molecule selected from the group consisting of interferon-$\alpha$, interferon-$\beta$, interferon-$\omega$, interferon-$\tau$, tumor necrosis factor-$\alpha$, tumor necrosis factor-$\beta$, interleukin-2, interleukin-7, interleukin-12, interleukin-15, B7-1 T cell costimulatory molecule, B7-2 T cell costimulatory molecule, immune cell adhesion molecule (ICAM) -1 T cell costimulatory molecule, granulocyte colony stimulatory factor, granulocyte-macrophage colony stimulatory factor, and combinations thereof.

FIG. 1A

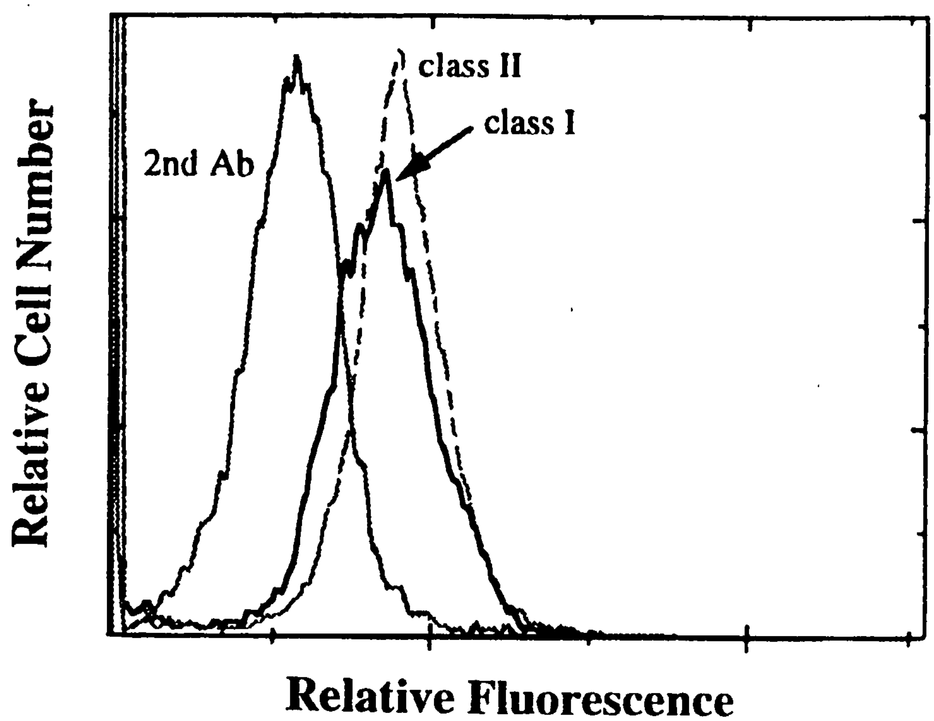

class II
class I
2nd Ab
Relative Cell Number
Relative Fluorescence

FIG. 1B

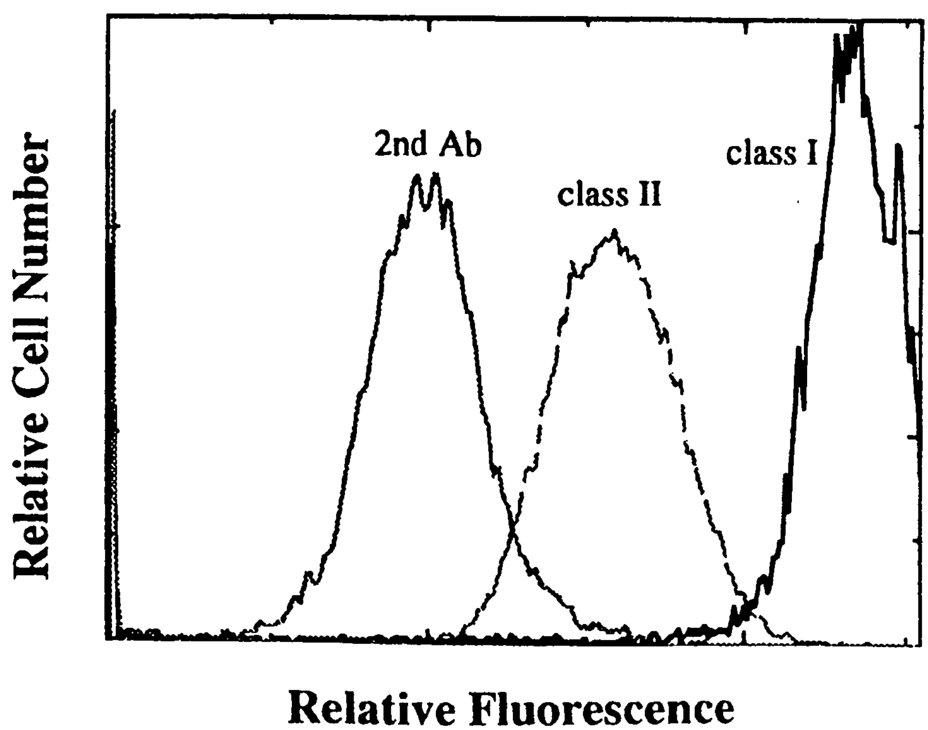

2nd Ab
class II
class I
Relative Cell Number
Relative Fluorescence

FIG. 2A

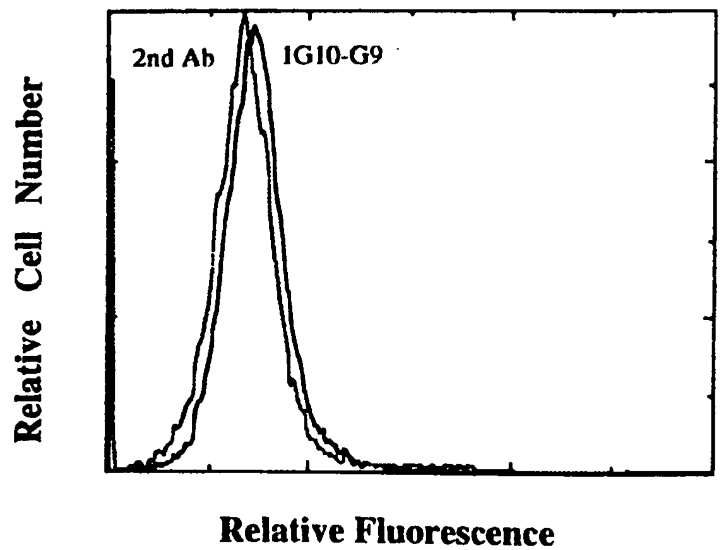



FIG. 2B

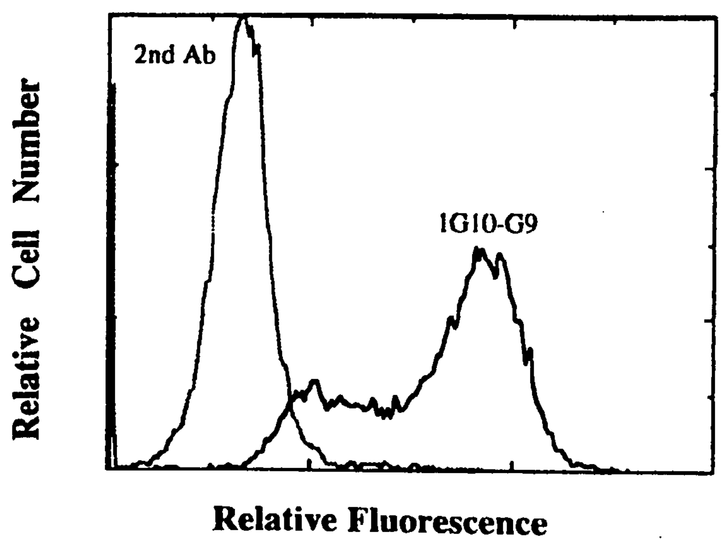



FIG. 2C

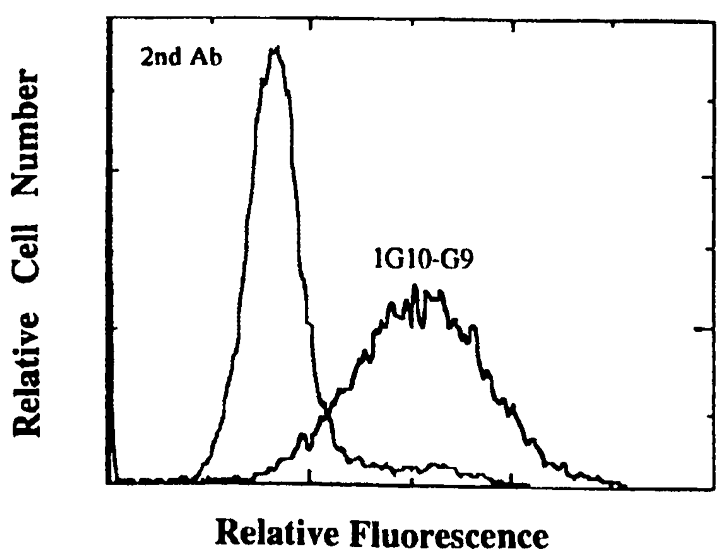


FIG. 3A

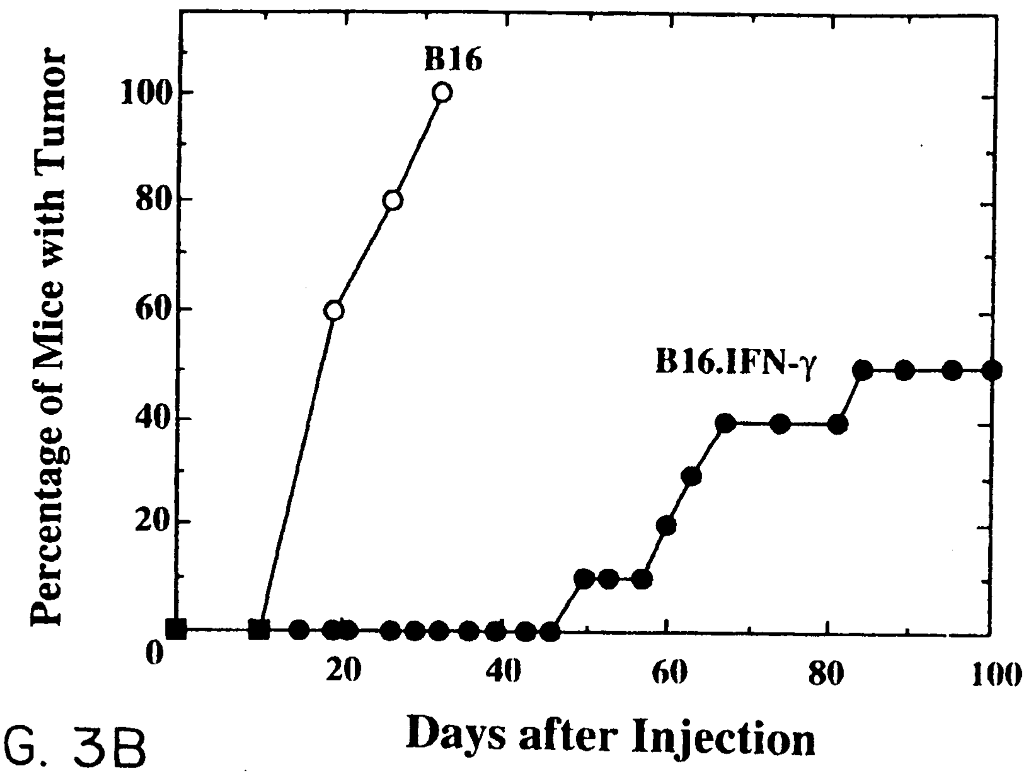

B16
B16.IFN-γ
Percentage of Mice with Tumor
0
20
40
60
80
100
20
40
60
80
100
Days after Injection

FIG. 3B

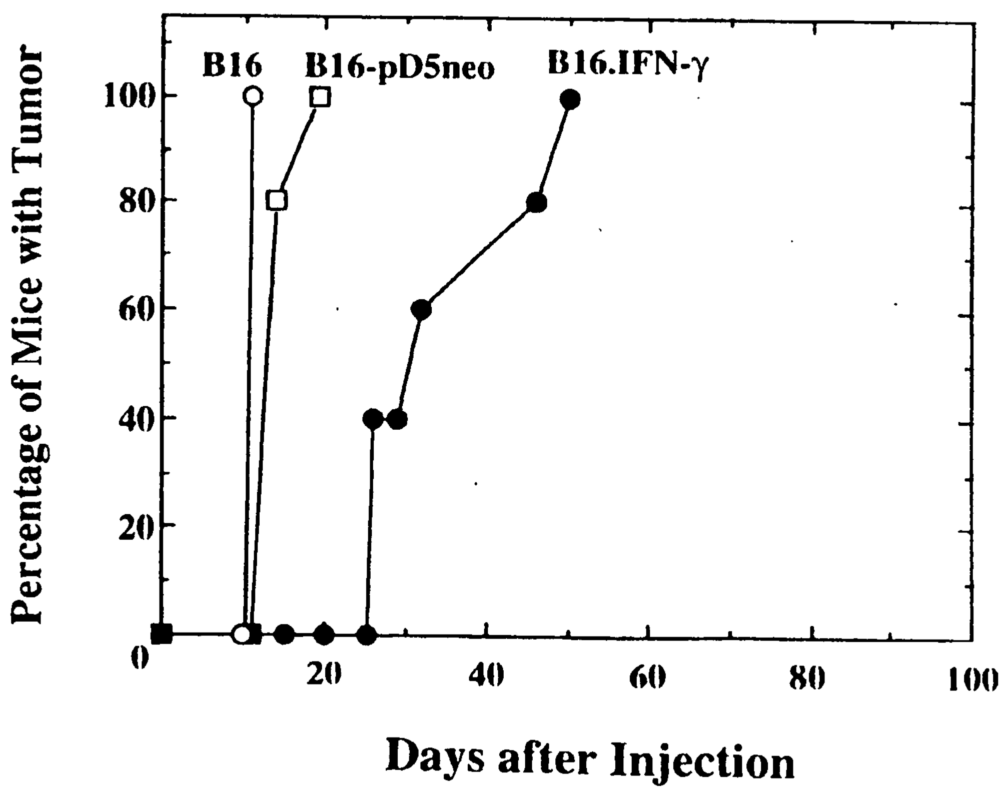

B16
B16-pD5neo
B16.IFN-γ
Percentage of Mice with Tumor
0
20
40
60
80
100
20
40
60
80
100
Days after Injection

FIG. 4

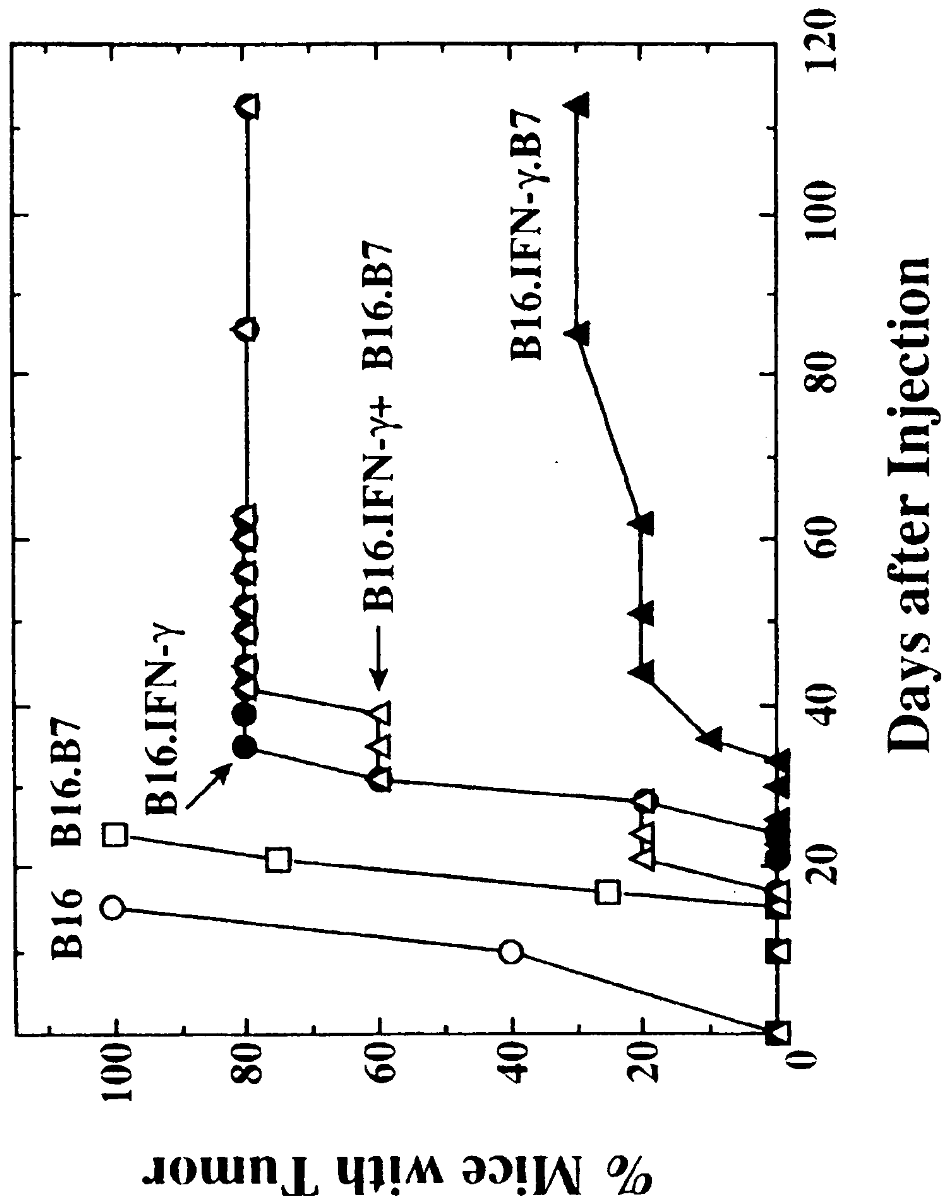

B16
B16.B7
B16.IFN-γ
B16.IFN-γ+ B16.B7
B16.IFN-γ.B7
% Mice with Tumor
Days after Injection
0
20
40
60
80
100
120

FIG. 5

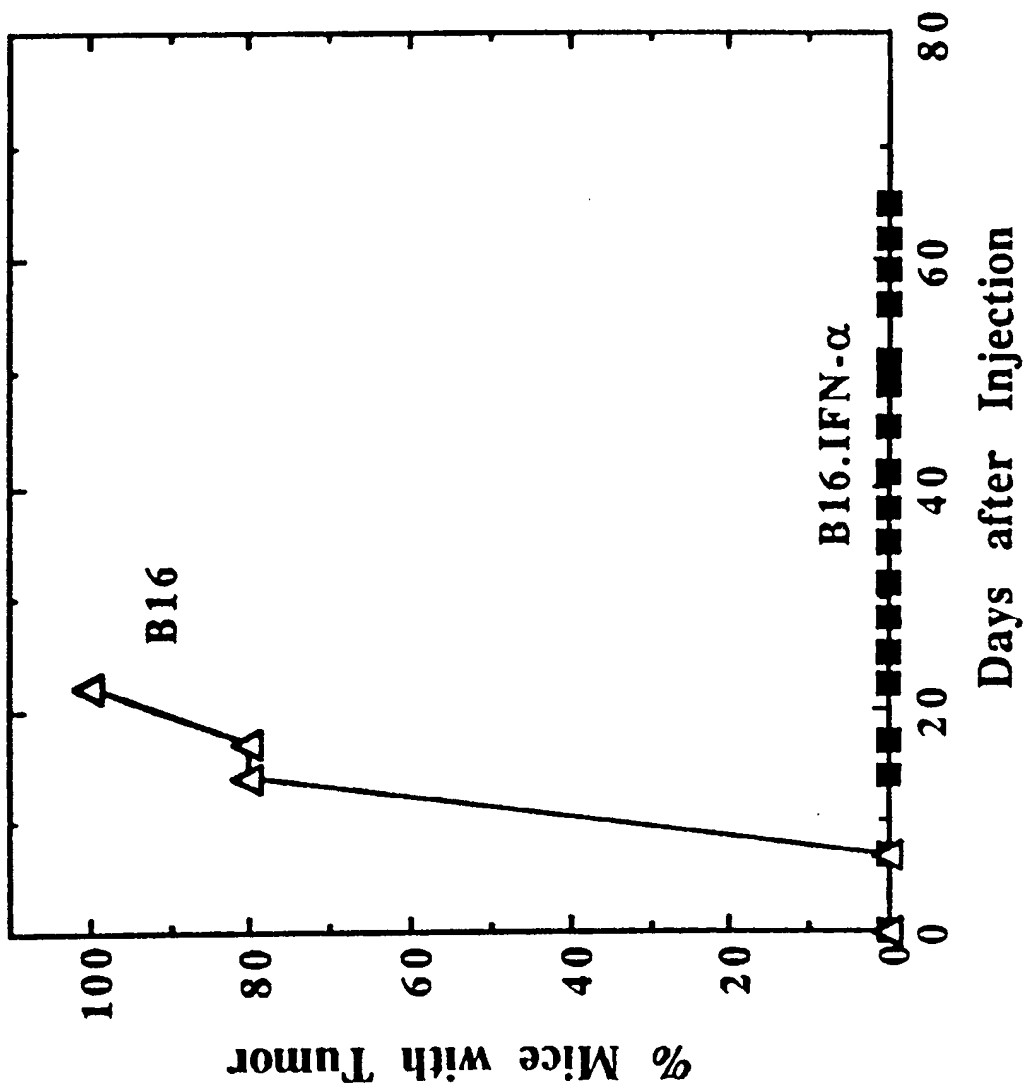

% Mice with Tumor
100
80
60
40
20
0
0
20
40
60
80
Days after Injection
B16
B16.IFN-α

FIG. 6

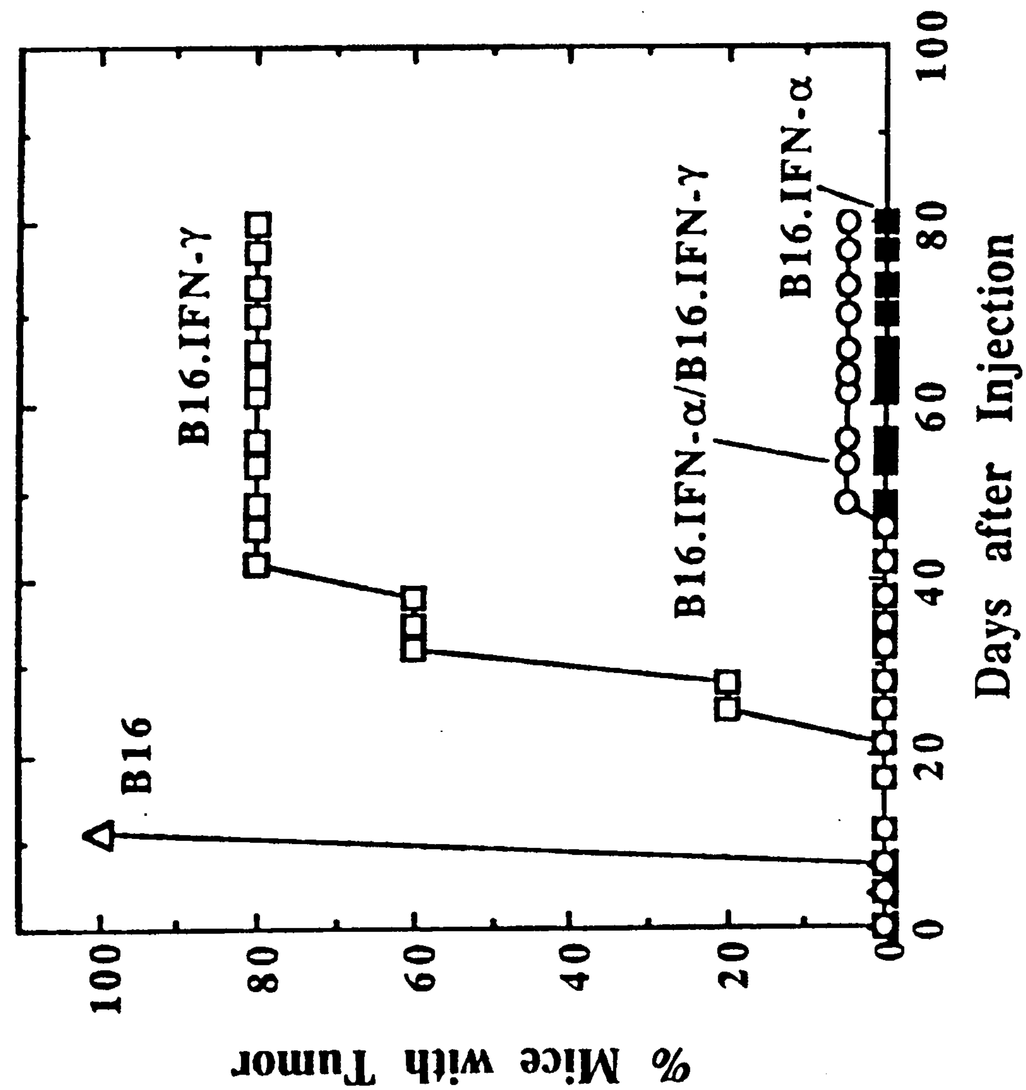

% Mice with Tumor
Days after Injection
B16
B16.IFN-γ
B16.IFN-α/B16.IFN-γ
B16.IFN-α
0
20
40
60
80
100

FIG. 7

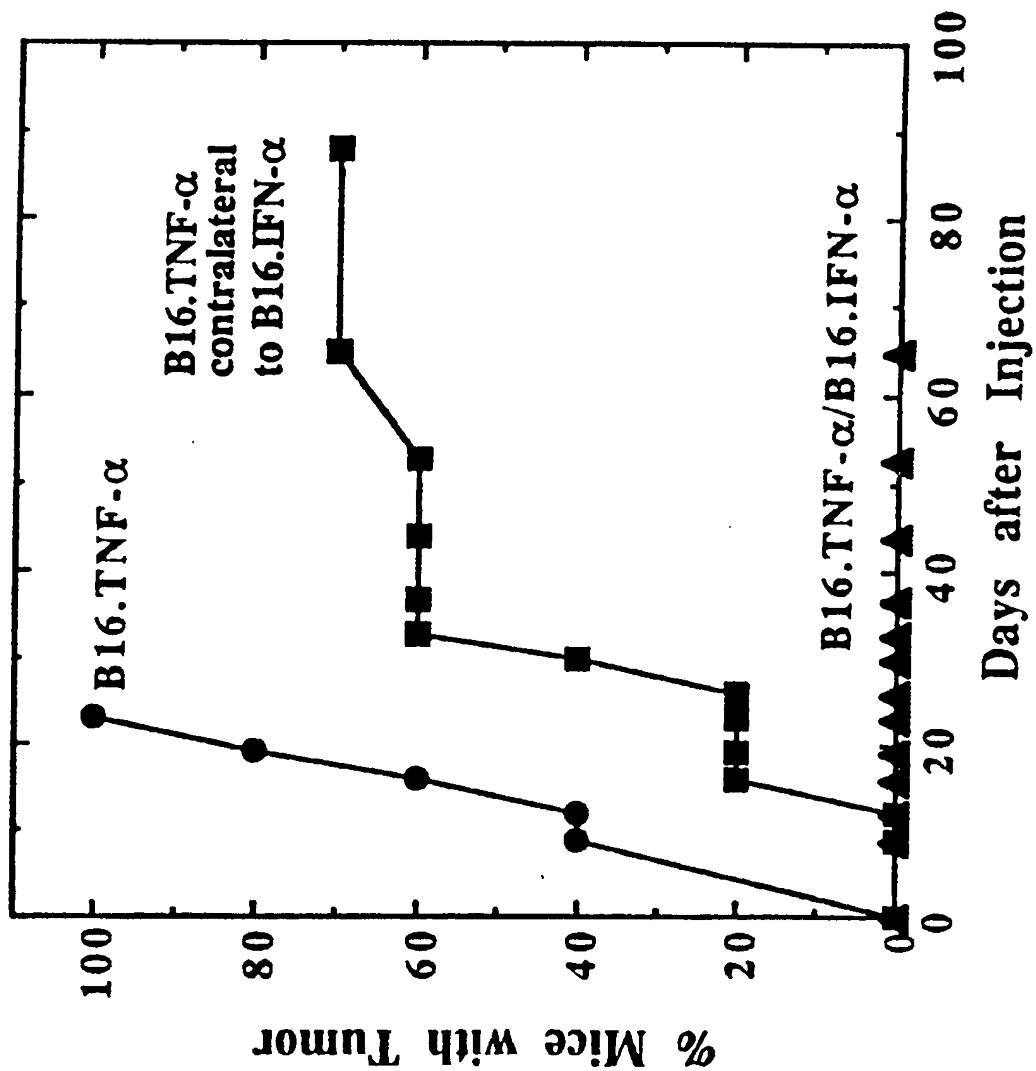

B16.TNF-α
B16.TNF-α contralateral to B16.IFN-α
B16.TNF-α/B16.IFN-α
% Mice with Tumor
Days after Injection
0
20
40
60
80
100

FIG. 8

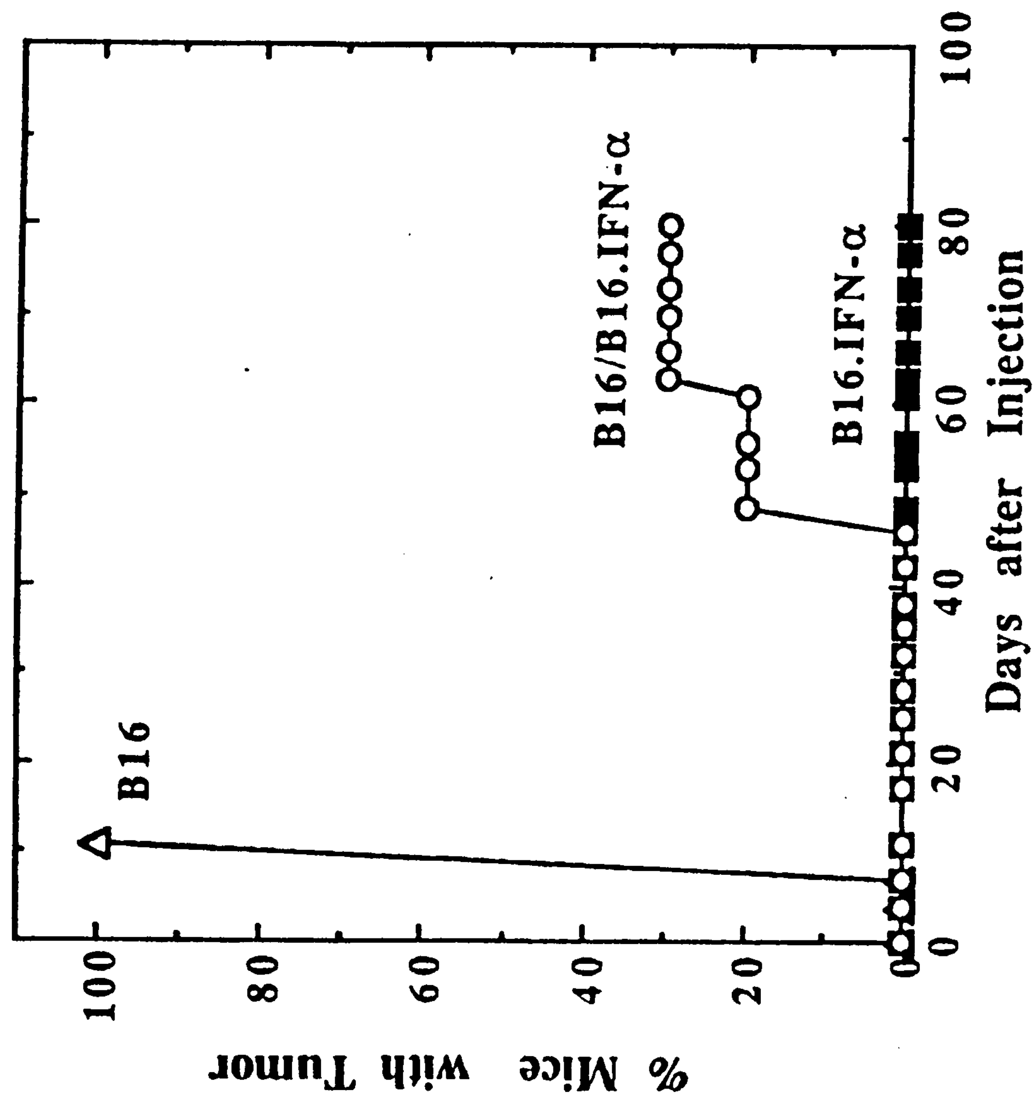

B16
B16/B16.IFN-α
B16.IFN-α
% Mice with Tumor
Days after Injection
0
20
40
60
80
100

FIG. 9

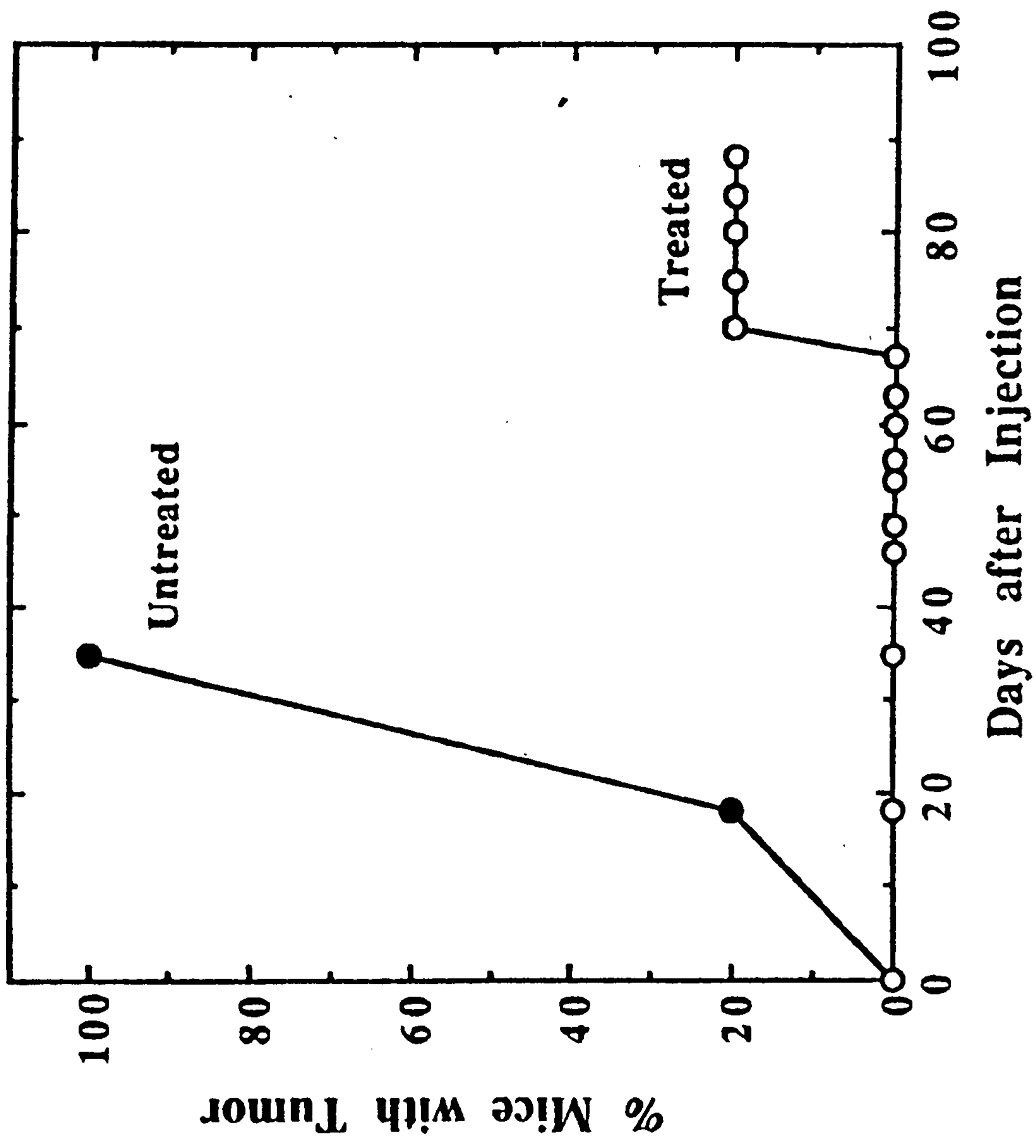

Untreated
Treated
% Mice with Tumor
Days after Injection
0
20
40
60
80
100

FIG. 10

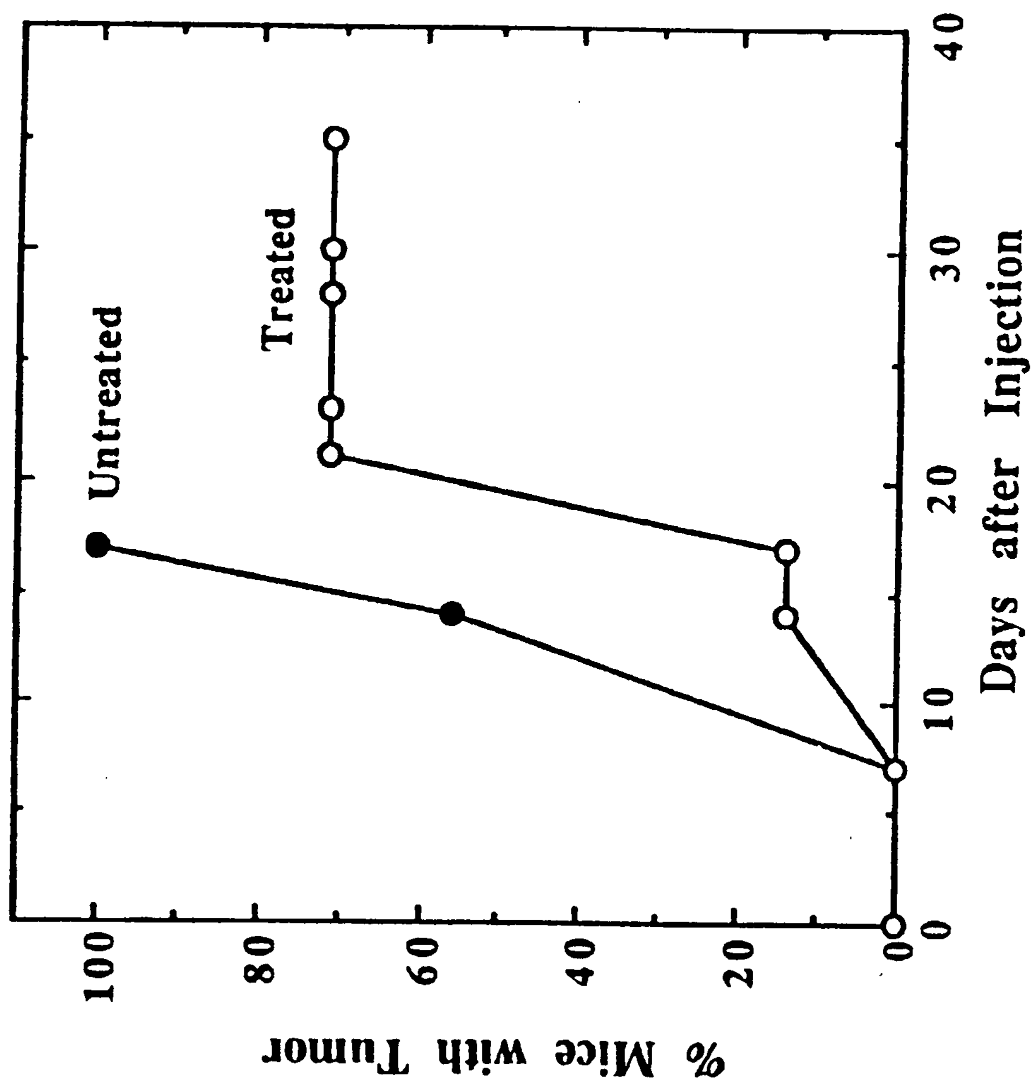

Untreated
Treated
% Mice with Tumor
0
20
40
60
80
100
0
10
20
30
40
Days after Injection

FIG. 11

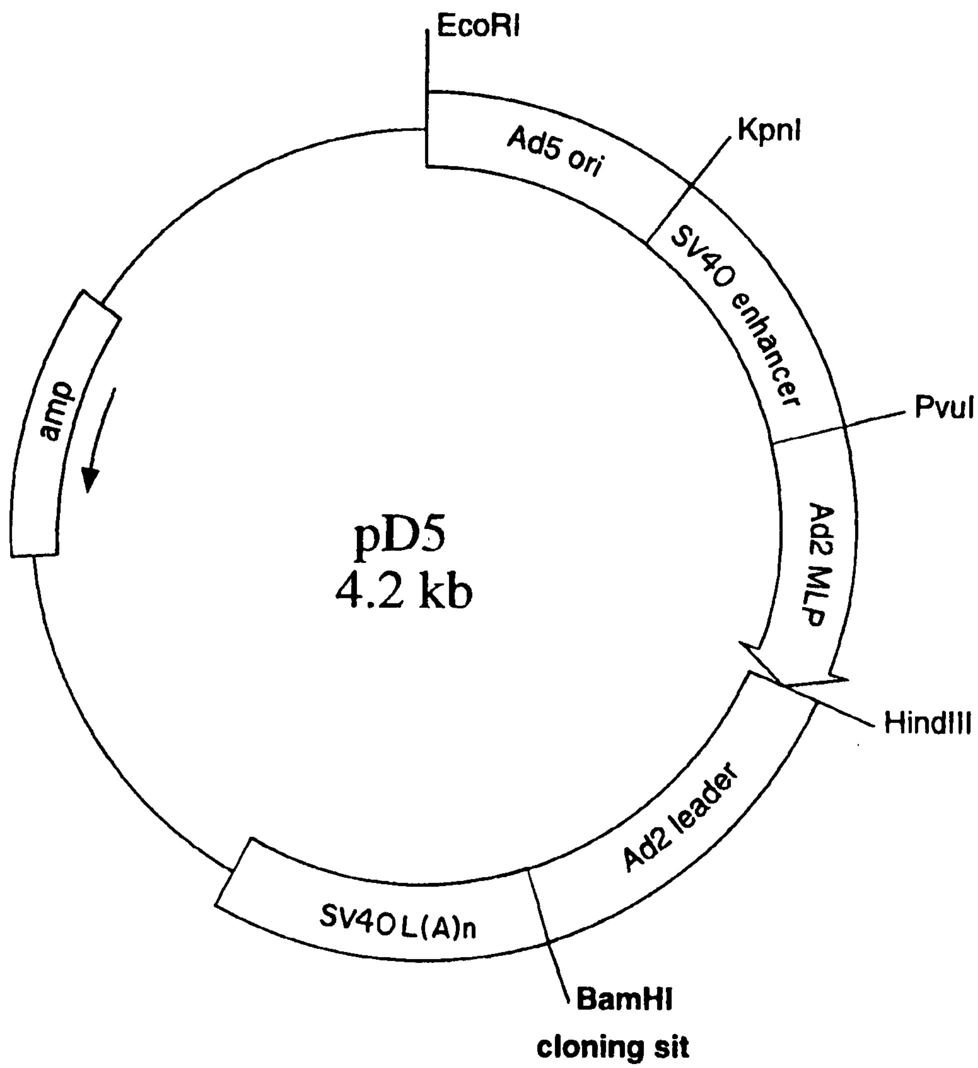
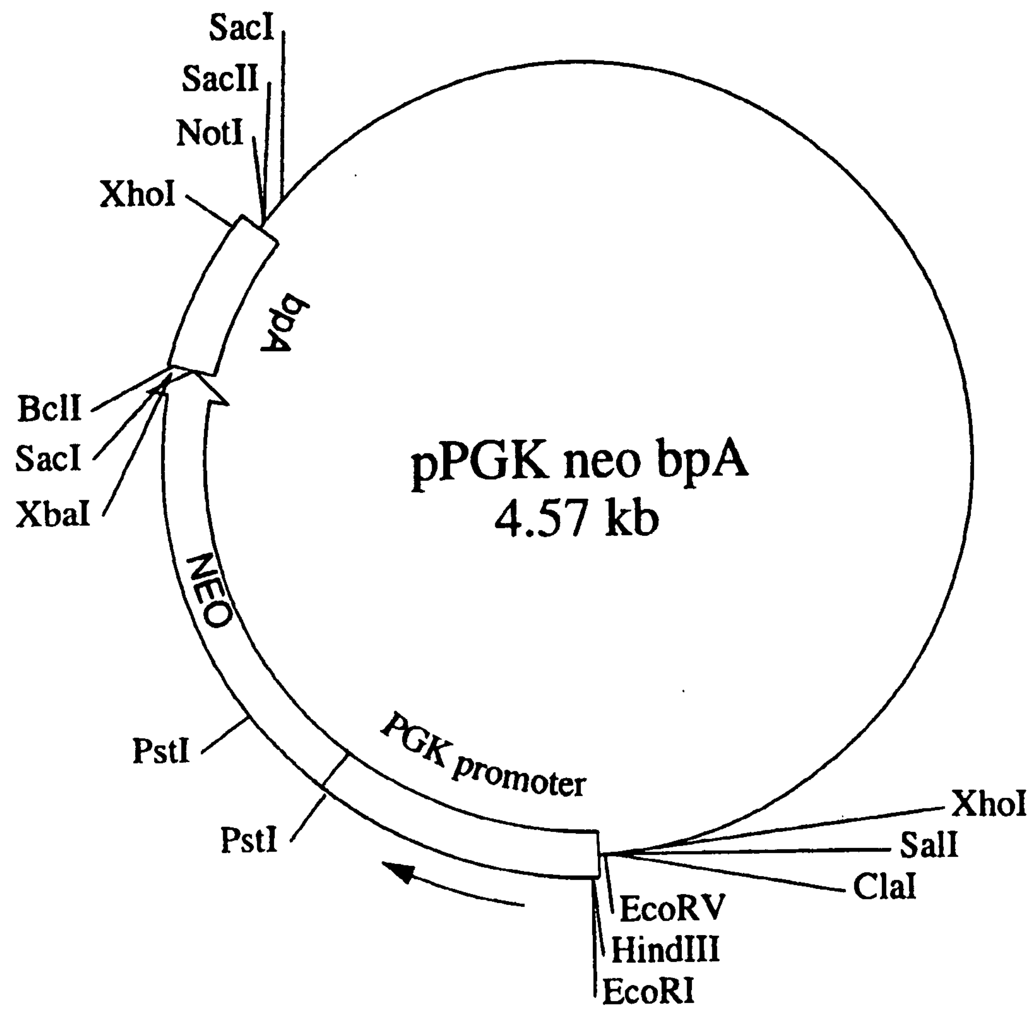

EcoRI
Ad5 ori
KpnI
SV40 enhancer
PvuII
Ad2 MLP
HindIII
Ad2 leader
SV40 L(A)n
BamHI
cloning sit
amp
pD5
4.2 kb

FIG. 12


SacI
SacII
NotI
XhoI
bpA
BclI
SacI
XbaI
pPGK neo bpA
4.57 kb
NEO
PstI
PstI
PGK promoter
XhoI
SalI
ClaI
EcoRV
HindIII
EcoRI

## INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 96/10502

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 6 A61K39/00 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO,A,94 21792 (VIAGENE INC) 29 September 1994<br>see page 2, line 31 - page 3, line 18<br>see page 6, line 17 - page 8, line 19<br>see page 36 - page 38; example 3<br>--- | 1-37 |
| X | WO,A,94 18995 (SLOAN KETTERING INST CANCER ;GANSBACHER BERND (US)) 1 September 1994<br>see page 12, line 4 - page 54, line 29<br>--- | 1-37 |
| X | WO,A,94 16716 (VIROGENETICS CORP) 4 August 1994<br>see page 13, line 17 - page 15, line 17<br>see page 170; example 30<br>see claims 1-22<br>---<br>-/-- | 20-37 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 4 October 1996 | 28. 10. 96 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. ( + 31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax ( + 31-70) 340-3016 | Sitch, W |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO,A,93 06867 (WHITEHEAD BIOMEDICAL INST ;JOHNS HOPKINS UNIVERSITY SCHOO (US)) 15 April 1993<br>see page 5, line 30 - page 8, line 20<br>see page 12, line 21 - page 13, line 18<br>see page 23 - page 34; examples 1-8<br>--- | 20-37 |
| X | WO,A,94 21808 (GENENTECH INC ;BOEHRINGER INGELHEIM INT (DE); BIRNSTIEL MAX L (AT)) 29 September 1994<br>see page 6, paragraph 4 - page 15, paragraph 1<br>--- | 20-37 |
| X | CANCER RESEARCH,<br>vol. 53, 1993,<br>pages 1107-1112, XP002015124<br>FERRANTINI ET AL: "ALPHA1-INTERFERON GENE TRANSFER INTO METASTATIC FRIEND LEUKEMIA CELLS ABROGATED TUMORIGENICITY IN IMMUNOCOMPETENT MICE:ANTITUMOR THERAPY BY MEANS OF INTERFERON-PRODUCING CELLS"<br>cited in the application<br>see page 1107,abstract<br>--- | 1,8,<br>15-17 |
| A | PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA,<br>vol. 86, 1989,<br>pages 9456-9460, XP002015125<br>WATANABE ET AL: "EXOGENOUS EXPRESSION OF MOUSE INTERFERON GAMMA CDNA IN MOUSE NEUROBLASTOMA C1300 CELLS RESULTS IN REDUCED TUMORIGENICITY BY AUGMENTED ANTI-TUMOR IMMUNITY"<br>cited in the application<br>see page 9456,abstract<br>--- | |
| A | CANCER RESEARCH,<br>vol. 52, 1992,<br>pages 4571-4581, XP002015126<br>OZZELLO ET AL: "CELLULAR EVENTS ACCOMPANYING REGRESSION OF SKIN RECURRENCES OF BREAST CARCINOMAS TREATED WITH INTRALESIONAL INJECTIONS OF NATURAL INTERFERONS ALPHA AND GAMMA"<br>cited in the application<br>see page 4571,abstract<br>---<br>-/-- | |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | CANCER RESEARCH, vol. 46, 1986, pages 3990-3993, XP000604846 BALKWILL ET AL: "HUMAN TUMOR XENOGRAFTS TREATED WITH RECOMBINANT HUMAN TUMOR NECROSIS FACTOR ALONE OR IN COMBINATION WITH INTERFERONS" cited in the application see page 3990,abstract | |
| A | FEBS, vol. 285, 1991, pages 199-212, XP000215712 FIERS: "TUMOR NECROSIS FACTOR.CHARACTERIZATION AT THE MOLECULAR,CELLULAR AND IN VIVO LEVEL" cited in the application | |
| P,X | INTERNATIONAL JOURNAL OF ONCOLOGY, vol. 7, no. 3, September 1995, pages 501-509, XP000605140 FLORES ET AL: "IFN-GAMMA AND B7 AS COSTIMULATORS OF ANTITUMOR IMMUNE RESPONSES" see the whole document | 20-37 |

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## INTERNATIONAL SEARCH REPORT

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 8-19,26-37
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although these claims are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/10502

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A-9421792 | 29-09-94 | AU-A- | 6411994 | 11-10-94 |
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| | | EP-A- | 0689593 | 03-01-96 |
| WO-A-9418995 | 01-09-94 | AU-A- | 6240594 | 14-09-94 |
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| | | FI-A- | 954383 | 18-09-95 |
| | | HU-A- | 73383 | 29-07-96 |
| | | JP-T- | 8507921 | 27-08-96 |
| | | NO-A- | 953684 | 18-09-95 |
| | | PL-A- | 311036 | 22-01-96 |